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Natural Compounds As Selective Inducers of Apoptosis In Human Melanoma Cells

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NATURAL COMPOUNDS AS SELECTIVE INDUCERS OF APOPTOSIS IN HUMAN
MELANOMA CELLS

By: Sudipa June Chatterjee

A Thesis

Submitted to the Faculty of Graduate Studies

Through the Department of Chemistry and Biochemistry

In Partial Fulfillment of the Requirements for

the Degree of Master of Science

at the University of Windsor

Windsor, Ontario, Canada

2010

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Natural Compounds As Selective Inducers Of Apoptosis In Human Melanoma Cells

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Abstract

Melanoma is the deadliest form of skin cancer notorious for its aggressiveness and chemo-resistance to standard anti-cancer therapy. Most of the current chemotherapeutic agents e.g. doxorubicin, cisplatin, taxanes and etoposide are highly toxic to non-cancerous cells. In this thesis my objective was to evaluate the effects of a new wave of natural products: pancratistatin (*Hymenocallis littorale*), dandelion root extract (DRE, from *Taraxacum Officinale*) and curcumin (from *Curcuma longa*) on chemo-resistant melanoma cells. I also investigated the mitochondria-targeting potential of tamoxifen (an estrogen antagonist) in sensitizing melanoma cells to the effects of pancratistatin and curcumin in combinatorial treatments. All the natural products are efficient in inducing apoptosis in melanoma cells. Interestingly, pancratistatin and curcumin were very effective when combined with tamoxifen (eliciting a synergistic response), and are non-toxic to normal fibroblasts. Therefore, this thesis provides evidence of potential anti-cancer therapies and non-toxic combinations to this deadly cancer.

Dedication

To my parents, for the unfaltering encouragement and support they have given me.

Acknowledgments

I would like to thank Dr. Pandey for all his support and mentoring, and for giving me the opportunity to do such gratifying work in this field. I would also like to thank my lab mates, past and present: Dennis, Pam, Katie and Lee-Anne. A very special thank you to Mallika, Carly, Vasanta and Corey for all your help, counsel and friendship. To the under-graduates of the Pandey lab - Phil, Pardis, Elie, Jeet, Parvati, Jess and Kate - I have appreciated all the help and friendship that you have provided over the past year. Thank you to all other graduate students in the department who have extended to me their assistance.

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List of Abbreviations

AIDS	Auto Immune Deficiency Syndrome
AIF	Apoptosis inducing factor
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
APAF-1	Apoptotic protease activating factor 1
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma-2
BSA	Bovine serum albumin
CC	Curcumin
CO ₂	Carbon dioxide
COX-2	Cyclooxygenase-2
DCFDA	2'-7'-dichlorofluorescein diacetate
DISC	Death inducing signaling complex
DNA	Deoxyribonucleic acid
DRE	Dandelion Root Extract
EDTA	Ethylenediaminetetraacetic acid
ER-	Estrogen receptor negative
ER+	Estrogen receptor positive
ETC	Electron Transport Chain
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	Fetal bovine serum
FDA	Food and Drug Administration
H ₂ O ₂	Hydrogen peroxide

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IAP	Inhibitors of apoptosis
IFN	Interferon
MDC	Monodansylcadaverine
MET	Metformin
MMP	Mitochondrial membrane potential
mtDNA	Mitochondrial DNA
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Narc	Narciclasine
NHF	Normal Human Fibroblast
NO	Nitric oxide
PBS	Phosphate buffer saline
PCD	Programmed Cell Death
PI	Propidium iodide
PQ	Paraquat
PST	Pancratistatin
RFU	Relative fluorescence units
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SMAC	Second mitochondria-derived activator of caspases
TAM	Tamoxifen
TNF	Tumour Necrosis Factor
TRAIL	TNF-related apoptosis inducing ligand
UV	Ultra-violet
VEGF	Vascular endothelial growth factor

Overview of the Thesis

A recent resurgence in the field of natural health products has brought to light new compounds/extracts with anti-cancer properties. Many standard chemotherapeutic agents, have been derived from natural sources, but are also toxic to normal cells. Moreover, singular treatment regimes of cytotoxic agents, immunotherapy, radiation or hormone therapy alone, have not been sufficient to completely eliminate the disease. However, combinatorial treatments of both have the potential to instigate an interminable response in tumourigenic cells, so that the disease cannot recur.

Chapter 1 is an introduction to this thesis and provides literature review for the work in subsequent chapters. It must be noted that all references are included right after each chapter.

The following 3 chapters will describe results for the respective projects, and each chapter is independent in terms of Introduction, Materials and Methods, Results and Discussion, as summarized below:

Chapter 2 examines the efficacy of the compound pancratistatin (*Hymenocallis littorals*) in inducing apoptosis in A375 and G361 human melanoma cells, as well as its combinatorial effect with tamoxifen. It also examines the effect of narciclasine (*Narcissus*) alone and in combination with tamoxifen in MCF-7 breast

cancer cell. Reference: Chatterjee SJ, McNulty J, Pandey S. Sensitization of human melanoma cells by tamoxifen to apoptosis induction by pancratistatin, a nongenotoxic natural compound. *Melanoma Res* 2010. E-publication.

Chapter 3 investigates the efficacy of dandelion root extract (DRE) in inducing apoptosis in A375 and G361 human melanoma cell lines. It also examines the effect of the anti-diabetes drug, metformin, to sensitize the relatively more resistant G361 melanoma to DRE.

Chapter 4 determines the effectiveness of the multi-faceted compound, curcumin (from *Curcuma longa*) in inducing apoptosis in A375 and G361 human melanoma cells. It also examines the effect of the estrogen receptor antagonist, tamoxifen, in sensitizing melanoma cells to curcumin. Furthermore, modes of cell death, attributed to curcumin and tamoxifen co-treatment, are also examined.

Chapter 5 provides overall conclusion and future prospectives for all three preceeding chapters.

Chapter 1

Introduction

Cancer

At a glance, the alarming cancer statistics for Canada predict 173, 800 new cases and a staggering 76, 200 deaths by the end of 2010 [1]. This translates to approximately 3,340 new diagnoses and 1, 470 deaths per week in Canada. Cancer incidences have been attributed to a multitude of factors, such as physical and chemical mutagens (e.g. UV radiation causing DNA damage), diet, smoking and even physical activity among other factors [2].

When healthy cells are faced with DNA damage they will try and repair the damage and activate checkpoints of the cell cycle. In effect the injured cell is protected. However, when the cell's protection parameters are overridden, it will then undergo apoptosis as a secondary response. In turn, the rest of the organism is protected from the probable erratic behaviour of one cell [3]. Characterized by uncontrolled cell proliferation, cancer cells lose this ability to commit suicide programmed cell death i.e. apoptosis.

Normal cell circuitry is deregulated in cancer cells through mutations in genes, such as proto-oncogenes, the result of which may cause over-production of growth stimulatory proteins [4]. On the other hand, over stimulation of pro-growth proteins is not enough to override the normal functioning of healthy cells. When safeguard mechanisms against growth-promotion fail, via mutated/non-functional tumour suppressors and other proteins, cells proliferate unchecked becoming tumourigenic [4]. Tumours share six characteristics, which differentiate them from normal cells: self-sufficiency (sustaining itself through excessive growth signals),

evading apoptosis, support through angiogenesis (bringing blood supply to tumour through vasculature), metastasis (spreading of the tumour into surrounding tissues) and invasion into tissues, unlimited replication ability and insensitivity to anti-growth signals [5].

Current anti-cancer therapies

The form of treatment administered to a patient will depend on two major criteria: the type of cancer, and the stage of tumour development. Cancer treatments include surgery, radiation, chemotherapy, immunotherapy, suicide genes and hormone therapy. All these treatment options result in the induction of apoptosis. The problems with current therapies are that the site of the malignancy may not be completely cleared of tumourigenic cells; and normal cells are exposed to these harmful treatments, which kill them.

Treatments can be categorized as either local (targeted treatment for tumours that have not yet spread) or systematic (broad range treatment for metastatic tumours). Local treatments will target tumours that are confined to a certain area, and are still in the early stages of development. On the other hand, systemic treatment involves therapy that will target cells that have metastasized throughout the body to other organs. Surgery is ideal for local treatment of a tumour, whereas chemotherapy covers a much broader area and would be the preferred option for metastatic tumours.

Chemotherapy and hormone therapy are used for the systematic treatment of cancer. In addition to this, systematic adjuvant therapies are also employed to

destroy any remnants of the tumour that have metastasized and could otherwise pose a recurring threat to the patient (especially with regards to breast cancer) [6]. Most anti-cancer drugs are genotoxic (targeting the DNA) or target the cell microtubule cytoskeleton (important for the formation of the mitotic spindle during cell division), thereby inducing apoptosis. Common anti-cancer chemotherapies include paclitaxel, cisplatin, vincristine and etoposide. Currently, anti-angiogenic therapy is receiving renewed interest. The protein vascular endothelial growth factor (VEGF) stimulates blood vessel vascularization. Anti-VEGF agents (e.g. bevacizumab) are being developed to prevent vascularization of blood vessels that support the tumour (e.g. metastatic breast cancer, non-small cell lung cancer and metastatic colorectal cancer). Cutting the blood supply to the tumour prohibits its survival [7].

Sometimes therapies, like radiation, render normal cells susceptible to tumourigenicity themselves. Both radiation and chemotherapy can cause oxidative damage (e.g. lipid peroxidation of membranes) to normal tissues. Currently, steps are being proposed to introduce anti-oxidants to combat this effect. However, this has proved to be detrimental to patients as tumour cells are also protected by these anti-oxidant supplements [8].

Therefore in a scenario to treat a patient with breast cancer, for example, the primary mode of treatment would involve localized treatment i.e. partial surgery of the region or possibly even a mastectomy. Radiation can be used post-mastectomy as a form of adjuvant therapy to remove any latent tumourigenic cells residing in the

surrounding lymph nodes or chest cavity [6]. If this fails, or the primary tumour has already metastasized, systematic therapy should be used as described above. If hormone therapy is used, its efficacy would rely on the expression of the cells' hormone receptors e.g. the estrogen receptor, and corresponding treatment to block this receptor with drugs, such as tamoxifen, would be employed. If the cancer has metastasized, chemotherapy such as taxanes (paclitaxel and docetaxel, which target microtubules) are used. Instead of using single, highly cytotoxic agents, for chemotherapy, new treatments are edging toward combinatorial therapies such as hormone therapy with chemotherapy or polychemotherapy, to reduce cancer tumours [6].

Apoptosis

Apoptosis is the Greek word for the “falling of leaves”, a phenomenon that is a physiological, continual, homeostatic event which maintains cell populations in the body, is required during embryogenesis, response to cellular injury (from toxic insults and disease) and self-tolerance of the immune system [9, 10].

Apoptosis is an energy-dependent process, which is also physiological and appropriate. This contrasts with necrosis (another form of cell death), which is an energy-independent, pathological process caused by injury [11]. Apoptosis is characterized by nuclear and cytoplasmic condensation (pyknosis), nuclear fragmentation (karyorrhexis) and formation of membrane bound apoptotic bodies [12]. When apoptosis go awry i.e. there is lack of control, excessive apoptosis may

result in neurodegenerative diseases (e.g. Alzheimer's disease), ischemia-related diseases (myocardial ischemia) and even autoimmune diseases (AIDS) [11]. Conversely, when cells ignore signals to commit suicide they evade apoptosis and can become tumourigenic. Apoptosis is triggered through two pathways: the extrinsic pathway and the intrinsic pathway.

The Extrinsic Pathway

The extrinsic pathway is also known as the death receptor-mediated pathway. This pathway uses Fas, TNF (tumour necrosis factor), TRAIL (TNF-related apoptosis-inducing ligand) receptors and their corresponding ligands to induce apoptosis. Stimulation by the ligand causes the corresponding receptor to cluster as it binds. The extrinsic pathway is facilitated by downstream families of proteins: caspases and sometimes the Bcl-2 family. In Fas-mediated apoptosis, for example, when the Fas ligand (FasL) binds to the receptor at the cell surface, it results in receptor-trimerization, after which the adaptor protein, Fas-associated death domain (FADD), binds to the receptor intracellularly [13]. FADD in turn recruits pro-caspase-8 from the cytoplasm [13]. The Fas-FADD-caspase-8 trinity forms a protein complex known as the death-inducing signaling complex (DISC) [14]. Apoptosis is then triggered in two ways: firstly, through a mitochondria-involved pathway, downstream of caspase-8 (initiator caspase); or secondly, through direct activation of caspase-3 (effector caspase) by caspase-8 [Figure 1].

In the indirect pathway, caspase-8, after binding to Fas associated death domain (FADD) [15], leads to downstream cleavage of BID to truncated BID (tBID). It is then tBID which causes the homo-dimerization of pro-apoptotic protein BAX (of the Bcl-2 family), which then translocates to the mitochondria [16]. After the mitochondria has been permeabilized (as described below- The Intrinsic Pathway), downstream auto-activation of caspase-9 and apoptosome formation follow, resulting in activation of the effector caspases -3, -6 and -7 [13] [Figure 1].

There have been reports that current chemotherapeutic drugs (doxorubicin, VP-16, daunorubicin and mitomycin-C) act to induce apoptosis in cancer cells through the activation of caspase-8, dependently and even independently of Fas [13].

Figure 1

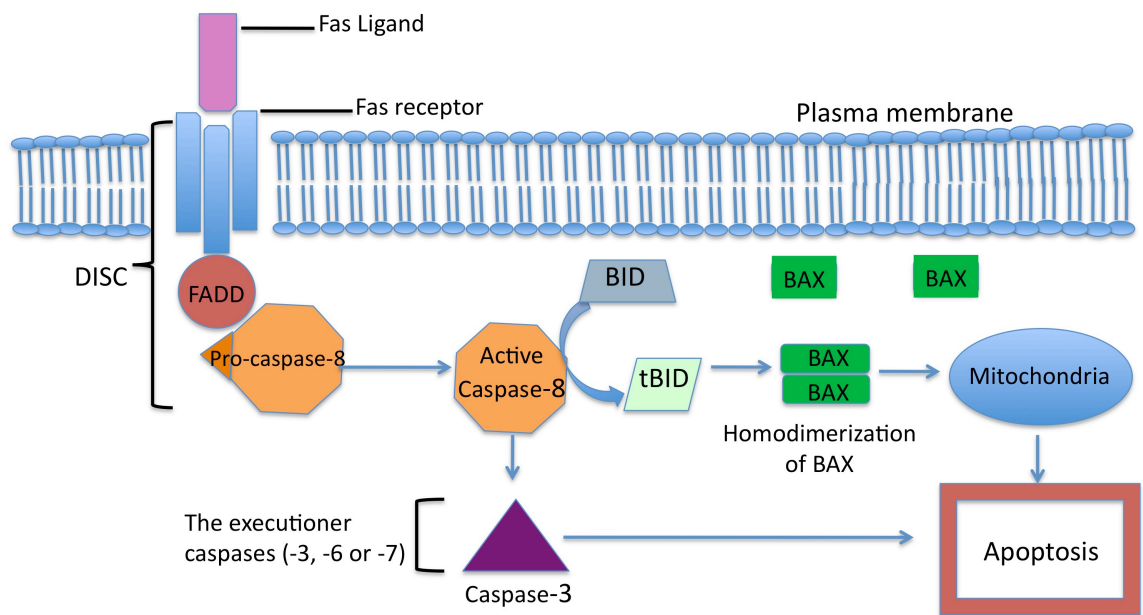


Figure 1: The Extrinsic Apoptotic Pathways. The figure indicates not only the extrinsic pathway (death receptor mediated) but also the cross-talk between apoptotic pathways associated with the mitochondria the two through the recruitment of Bid.

The Intrinsic Pathway

The intrinsic pathway is also known as the mitochondria-dependent pathway. Central to this pathway is the Bcl-2 (B- cell leukemia/lymphoma 2) family of proteins. The family consists of the pro-apoptotic proteins (BID, BAX, BAK, BIM) and the anti-apoptotic proteins (Bcl-2, Bcl-xL). The intrinsic pathway is activated by factors including oxidative stress, DNA and cytoskeletal damage, resulting in transcription or post-translational activation of pro-apoptotic proteins of the Bcl-2 family. Upon stimulation, these proteins sequester the anti-apoptotic Bcl-2 proteins allowing the pro-apoptotic proteins, such as BAX, to act on the mitochondria. This causes the release of pro-apoptotic factors from the mitochondria like, cytochrome *c*, SMAC/DIABLO and apoptosis inducing factor (AIF) [14]. When cytochrome *c* is released it associates with the apoptotic protease activating factor-1 (APAF-1) and pro-caspase-9 to form an apoptosome, which then activates the executioner caspases (-3, -6 and -7) thereby committing the cells to apoptosis [18]. Other than the roots of its activation, this mitochondria-dependent pathway overlaps with the extrinsic pathway, resulting in a link between both modes of apoptosis (through the pro-apoptotic protein Bid) [Figure 2].

Figure 2

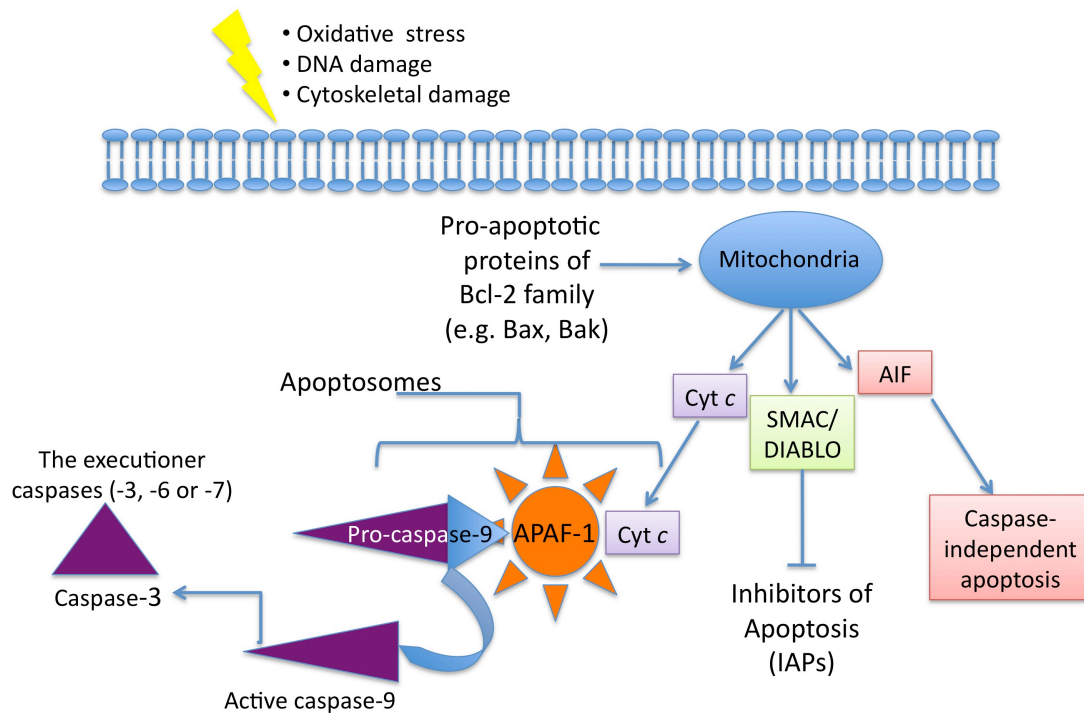


Figure 2: The Intrinsic Apoptotic Pathway. The pathway highlights how cellular/environmental stress factors can result in the recruitment of pro-apoptotic members of the Bcl-2 family to incite mitochondrial permeabilization. This causes the release of other pro-apoptotic proteins from the mitochondria, eventually leading to apoptosis.

Autophagy

A second form of programmed cell death (PCD) is autophagy. Autophagy has been classified as PCD II and is unlike apoptosis, which falls into the category of PCD I. This evolutionary process has been named from the Greek term for “self-eating” (auto = “self” + phagy = “eat”). Double-membranes form a vesicle, known as the autophagosome, and this structure surrounds cytoplasmic organelles and proteins, thereby engulfing or “eating” the structures [19]. The autophagosome then fuses with a lysosome (containing digestive enzymes), forming an auto-lysosome, degrading the pre-engulfed cytoplasmic contents [19]. The process occurs when the cell is under environmental stresses such as nutrient deprivation, hormonal imbalance, high temperature, hypoxia, and even under intracellular stresses of misfolded proteins and impaired organelles [19] [Figure 3]. Autophagy can either be a pro-survival strategy for cells to adapt to stress, or if the stress is massive enough, autophagy can be a pro-death mechanism whereby the cells die. Autophagic cell death occurs without the activation of caspase enzymes. There are various forms of chemotherapy that have been shown to induce pro-survival autophagy. This causes cancers to become chemo-resistant to the treatment, as the cells turn to survival autophagy to adapt to the stress created by these anti-cancer drugs, and thus unintentionally granting them survival [20]. Therefore, autophagy can result in either a pro-survival or a pro-death response depending on the intensity of the cytotoxic insult.

Figure 3

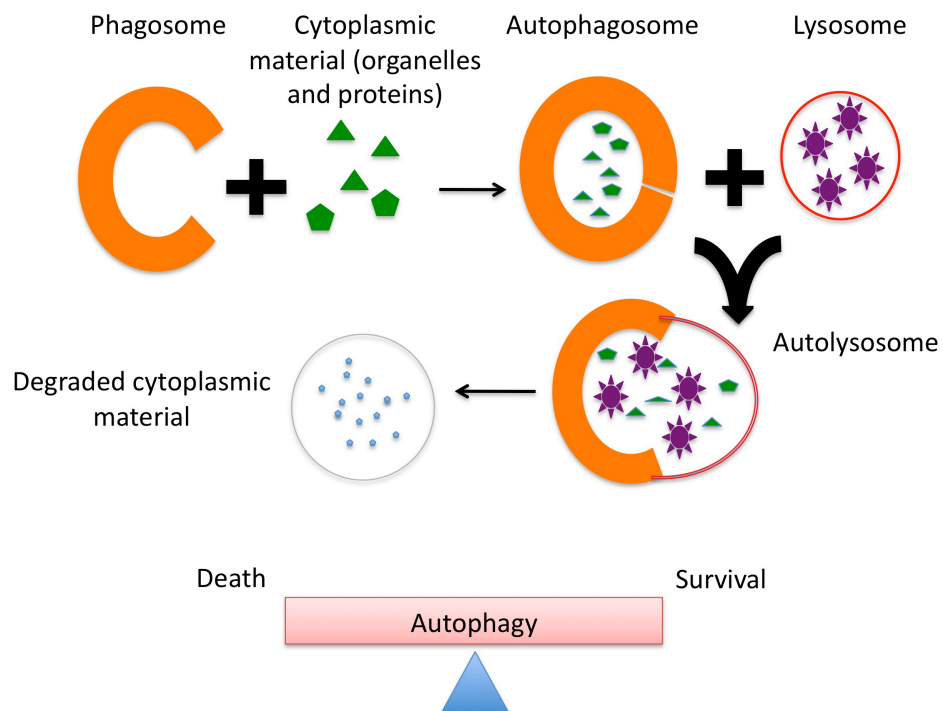


Figure 3: The Process of Autophagy. The figure shows how environmental stress factors induce autophagy. Importantly, degraded cytoplasmic material can either result in pro-death or pro-survival of the cell. Pro-survival autophagy is one of the ways cells adapt to the cytotoxic insults from chemotherapy and in this way can cause resistance to that mode of treatment.

Programmed Cell Death (PCD) and Cancer

By nature, cancer is elusive to a spectrum of therapies, since it ignores the signals to commit suicide (apoptosis). Traditionally, cancer therapeutic drugs have targeted the microtubules or have been genotoxic (DNA-targeting). Circumventing apoptosis activation allows tumourigenic cells to survive, proliferate and also muster resistance against these anti-cancer drugs [5]. In addition, these drugs target normal cells resulting in non-tumourigenic fatalities. To effectively target only cancer cells the factor that renders them contrastive must be exploited. It is the difference in bioenergetics that sets tumourigenic cells apart from normal cells, and at the centre of this phenomenon lies the mitochondria. Mitochondria function to generate ATP for the cell, which the cell can use in its many energy-driven, biochemical processes. Warburg hypothesized that cancer cells rely more on glycolysis than oxidative-phosphorylation to meet their energy requirements [21]. Electron microscopy has confirmed that indeed, tumour cells not only have fewer mitochondria, they appear morphologically different and also function abnormally compared to normal cells [22]. Therefore, mitochondria being a central component of apoptosis and the cellular bioenergetics hub, makes them a vulnerable target in cancer cells, and if directly targeted, will help by-pass the problem of cancer-cell resistance to treatment [23].

Moreover, non-apoptotic cell death pathways, such as mitotic catastrophe, and necrosis, have been implicated in tumourigenesis [24]. Autophagy as mentioned before has both pro-survival and pro-death character, but regardless of this duality,

autophagy is also another form of non-apoptotic cell death. Cancer cells express lower levels of autophagic proteins like Beclin1, compared to normal cells [25]. For example, the autophagy-related gene Beclin 1 (BECN1) deleted in 75% of ovarian cancers, 50% of breast cancers and 40% of prostate cancers, and has reduced expression levels in cervical, brain, hepatocellular and colon cancers [20]. When Beclin1 is disrupted it promotes tumourigenesis [25]. When it is overexpressed, BECN1 can inhibit tumourigenesis [26]. Since expression of autophagy-related genes is either null or suppressed in cancer cells, it is thought that autophagy could play a role as a tumour suppressor [20].

There are interactions between the autophagic and apoptotic pathways, but the connection between these two pathways is not yet understood. It is possible however, that once the targets are determined for autophagic death-induction, drugs may be generated to target this non-apoptotic pathway in cancer cells [24].

Melanoma

Melanoma is the deadliest form of skin cancer and known to be notoriously chemoresistant and very aggressive. In 2009, Meyle and co-workers, reported that melanoma incidences currently stand at 1 in 50 people in Western populations [27], and has become the second most common cancer in young Canadian adults. This cancer has been proven to be resistant to radiotherapy, chemotherapy, immunotherapy and hormonal therapy [28]. At present the primary treatment for melanoma is surgery, which has a success rate greater than 95% with complete

recovery following it [29]. However, surgery is only effective when the melanoma lesions are detected in their earliest stages of development. If undetected the cancer will spread rapidly, and will not be contained by any form of chemo-, immuno- or radiotherapies. Currently the only FDA approved drug available to treat malignant melanoma is dacarbazine. However, it has a response rate between 15-25% in clinical trials [30]. With such dismal statistics, the Dartmouth regimen was proposed, which combined dacarbazine with cisplatin, carmustine and tamoxifen (estrogen receptor antagonist), and it was found that this combination did not confer any extra advantage compared to dacarbazine itself at stage IV of the disease [31].

It was concluded that standard chemotherapeutics ranging from platinum drugs, hormonal mimics (tamoxifen), the natural compound families such as epipodophyllotoxins (namely, etoposide), vinca alkaloids and taxanes to anthracyclines (e.g. doxorubicin), cyclophosphamide and triazenes were all ineffective against chemoresistant melanoma [29].

Natural compounds

Pancratistatin

Pancratistatin (PST) is a natural compound isolated from the Hawaiian spiderlily (*Pancratium littorale*). It was first isolated in 1992 and has shown abilities as a cytostatic (halting cell growth) and anti-neoplastic compound [32, 33, for structure see *Appendix*]. However, we are only beginning to understand what the possible mechanism for PST might be. Our lab has previously shown that PST has successfully induced apoptosis in a variety of cancer cells: Jurkats (human lymphoma), SHSY-5Y (human neuroblastoma), NT-2 (human teratocarcinoma) MCF-7 (breast carcinoma) and 5123tc (rat hepatoma) [34]. Not only has the effect been observed at low doses but the normal non-cancerous cell counter-parts normal human fibroblasts (NHF) do not experience any PST-induced toxicity. This indicates that PST is specific in targeting cancer cells. Furthermore PST shows no structural similarity with any DNA-targeting drugs such as the class of anthracyclines (e.g. doxorubicin, daunorubicin, idarubicin).

Upon inspection of its mode of action, it was observed that PST did not target DNA in human neuroblastoma cells (SHSY-5Y), and resulted in an increase in the level of caspase-3 activation and generation of reactive oxygen species (ROS). This was accompanied by a decrease in adenosine- 5'-triphosphate (ATP) and dissipation of the mitochondrial membrane potential [35]. In human lymphoma (Jurkat) cells, PST showed greater specificity in apoptosis induction compared to paclitaxel and VP-16 [36]. In a previous study PST also induced apoptosis in estrogen receptor

positive (ER+) and estrogen negative (ER-) breast cancers cells, a probable target being mitochondria, as observed by increased production of ROS in both cell lines. ROS production further increased upon combination with the widely-used breast cancer drug tamoxifen, leading Siedlakowski *et al.* to infer that the co-treatment resulted in synergy between the two compounds, since both compounds most likely target mitochondria-related pathways [37].

Narciclasine

Narciclasine (Narc) is an *Amaryllidaceae* isocarbostryl compound from the plant *Narcissus*. It acts as a plant growth regulator and was used by the ancient Greeks to treat cancer [for structure see *Appendix*]. Structurally it is very similar to PST with the exception of one hydroxyl group [38]. It has already proven its efficacy as a cytotoxic agent *in vitro* in murine leukemia cells and in various human cancer lines [39], and similar work has been conducted to that of the previous work conducted with PST (from our lab using neuroblastoma) [35] by Dumont and co-workers and Ingrassia co-workers. Both these groups have tested narciclasine and its derivatives on a variety of different cancer lines and have reinforced the effectiveness of this compound [40, 41]. It is thought that narciclasine can target the actin organization (the cytoskeleton of the cell) of glioblastoma multiforme cells [42].

Dandelion Root Extract (DRE)

Taraxacum Officinale is most commonly known as dandelion. This garden weed has been used as a detoxifying herb in traditional Chinese medicine, for ailments ranging from digestive disorders to complex disorders such as uterine, breast and lung tumours [43]. While Native Americans have harnessed their properties to cure indigestion, heartburn and kidney disease, traditional Middle Eastern remedies have used dandelions for liver and spleen ailments, [43, 44]. In the 16th century, dandelion (*Taraxacum officinale* Weber ex Wiggers) was used by East Indians as a hepatic stimulant, diuretic, for other liver disorders, and most interestingly, for chronic skin diseases [45, 46].

Scientific advancements have been made using this common weed in the area of anti-cancer natural health products. Recently, Jeon and co-workers, have shown that ethanolic *Taraxacum Officinale* extracts and derivative forms thereof, reduce levels of reactive oxygen species (ROS), nitric oxide production (NO) and inhibit COX-2 expression or its anti-oxidant activity, thus making dandelion extracts not only anti-inflammatory, but anti-angiogenic, anti-nociceptive (preventing pain) and also anti-carcinogenic [47]. Regardless of the valuable traditional knowledge of *Taraxacum* anti-tumor activity, there has been inadequate biochemical research to apply this knowledge to cancer cell lines and especially chemo-resistant melanoma.

Curcumin (CC)

This multi-faceted compound comes from the turmeric spice, *Curcuma longa*, a member of the ginger family [for structure see *Appendix*]. Traditionally used in Southeast Asian medicine, and especially Ayurvedic medicine, curcumin's capabilities as an anti-rheumatic, treating disorders related to diabetic wounds, hepatic disorders, respiratory problems and anorexia among other ailments, has drawn much attention to this common compound [48]. Furthermore it has gained recognition as a "magic-bullet" with regards to its anti-inflammatory, anti-oxidant, anti-microbial, hepatoprotective and anti-carcinogenic activity [48, 49, 50].

Much scientific advancement has been made proving curcumin's ability as a worthy chemotherapeutic. For instance, CC has been shown to effectively induce apoptosis in HepG2 hepatocellular carcinoma, MCF-7 and MDA-MB breast carcinoma [51]. Of its many targeted pathways, curcumin treatment resulted in apoptosis induction, which was associated with the generation of reactive oxygen species (ROS) in HT-29 and HCT116 colon cancer cells, Hep 3B and HepG2 hepatomas, human submandibular gland carcinoma (HSG) and even a transformed human skin cancer cell line (COLO16) [52-55]. Regardless of the positive evidence underlining its anti-carcinogenic activity and its multitude of targets, the mode by which curcumin actually commits cancer cells to death is still not understood.

Synthetic compounds

Tamoxifen

Tamoxifen (TAM) is a breast cancer drug that has been used in the past 30 years as an effective pharmaceutical combating metastatic breast cancer; it has been used as adjuvant therapy [for structure see *Appendix*], preventing the recurrence of the tumour in the mammary tissues [56, 57]. It has also been used to treat other cancers such as ovarian, pancreatic, hepatocellular, renal cell carcinoma and malignant melanoma and gliomas [58].

Originally, tamoxifen's mode of action was thought to be through the inhibition of estrogen receptors, thereby blocking growth factors that otherwise sustain estrogen receptor (ER) positive breast carcinoma, resulting in growth cessation of the cells [59]. However, there has been clear indication that TAM has extended its cytotoxic effects to ER negative breast cancer cells through the targeting of mitochondria [37, 59].

TAM was found to cause the collapse of the mitochondrial membrane potential, target the electron transport chain (ETC) of the mitochondria and act as an uncoupling agent [58, 60]. Previous studies have shown that it can inhibit complex II and to a lesser extent complex IV of the ETC of rat liver mitochondria [60]. More recently, Moreira *et al.*, reported that tamoxifen also targets complex I [61]. Besides targeting the mitochondria, it is thought that TAM also targets protein kinase C, ceramide, JNK and p38, caspases (-3, -8 and -9), lipid bilayer membranes, calmodulin and TGF- β [58].

Tamoxifen's efficacy as a single agent treatment was received with disappointing results. In a phase II study, patients with malignant melanoma were administered with high doses of tamoxifen, which showed that it was ineffective in these patients, even resulting in moderate to severe nausea in some [62]. Soengas and Lowe have classified tamoxifen as an agent to which human melanoma cells are resistant *in vivo* [29].

Metformin

Metformin is from the class of biguanide drugs. This drug primarily reduces production of hepatic glucose in the system thus preventing hyperglycemia in the body [63]. Not only does it prevent the aforementioned glucose production, but also reduces levels of non-esterified fatty acids and triglycerides in the plasma [63]. There have been studies conducted with a sample population of diabetics, which have proven a correlation between diabetic patients taking metformin and a reduced incidence of cancer and lower rates of mortality among them [for structure see *Appendix*]. In a study by Jiralerspong and co-workers, this was clinically proven when it was found that diabetics with breast cancer taking metformin, had a higher pathological complete response than diabetic breast cancer patients not taking the drug [64]. Healthy, normal cells express adenosine monophosphate-activated protein kinase (AMPK), which is directly downstream of the tumour suppressor LKB1, and is usually activated as a reaction to cellular energy stress. Metformin activates AMPK, resulting in the blockage of ATP consuming pathways (e.g.

gluconeogenesis) and consequent activation of the ATP generating pathways (e.g. glycolysis). AMPK activation has been shown to inhibit two gluconeogenesis-promoting genes – phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase)- though the full mechanism has not been established yet [63]. How metformin affects cancer cells is not understood, though it is clear that cancer cell energy metabolism differs from that of normal cells, and this difference makes tumourigenic cells vulnerable to metabolism interfering drugs like metformin [65].

Objectives

Based on the literature provided, the objective of this thesis is: firstly, to determine the efficacy of pancratistatin on A375 and G361 human melanoma cells, and determine whether the estrogen antagonist tamoxifen (known to also directly target the mitochondria) can sensitize these cells further to the effects of PST to instigate a possible synergistic apoptotic response; secondly, to evaluate the potential of dandelion root extract as an anti-cancer agent in A375 and G361 human melanoma cells; lastly, to investigate the effect of curcumin and tamoxifen treatment on A375 and G361 melanoma cells. For all these objectives the mechanisms by which cell death occurred were also examined.

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Chapter 2

Sensitization of human melanoma cells by tamoxifen to apoptosis induction by pancratistatin, a non-genotoxic natural compound

Abstract

The objective of this study was to determine the efficacy of the natural compound Pancratistatin (PST), isolated from the *Hymenocallis littoralis*, to induce apoptosis in human melanoma cells. Melanoma is an aggressive form of skin cancer that is commonly fatal if not diagnosed in its early stage of development. Melanoma is resistant to many treatments, thus drastically limiting chemotherapy options for this cancer. We have shown that exposure to PST induces apoptosis in human melanoma within 72 hours using Hoechst staining. Interestingly Tamoxifen (TAM), an estrogen receptor antagonist, sensitizes these cells to apoptosis induction by PST as observed with Hoechst and Annexin-V staining. This co-treatment did not affect the viability of normal non-cancerous human fibroblasts. Both of these compounds (0.5 μ M PST and 5 μ M) have been shown to target the mitochondria synergistically, as indicated by higher levels of reactive oxygen species (ROS) generation from isolated mitochondria. PST alone and in combination with TAM shows depolarization of the mitochondrial membrane potential as demonstrated by JC-1 staining (0.5 μ M PST and 5 μ M). Melanoma drug resistance was not observed after post-treatment recuperation, as cells displayed apoptotic morphology up to 96 hours after drug-free media replacement. Our results indicate that TAM alone does not induce apoptosis in this cell line, but sensitizes the mitochondria, thereby enhancing the effect of PST exposure. In conclusion, combination of two non-genotoxic compounds offers a novel treatment regime for this notoriously resilient form of skin cancer.

Introduction

Melanoma is considered the most aggressive form of skin cancer and one that is reputed for its hallmark chemoresistance such that it eludes a variety of current chemotherapies [1,2]. Cytotoxic courses of treatment, such as chemotherapeutic drugs, radiation, immunotherapy and also suicide genes have been used to induce apoptosis in melanoma, increasing survival by only a few months [3]. Most chemotherapies and radiation therapies induce apoptosis by targeting DNA and/or DNA repair mechanisms of the cancer cell. Recently, natural compounds have been shown to induce apoptosis e.g. etoposide, a genomic drug that inhibits topoisomerase II [4]. Taxol, another natural compound used as a chemotherapeutic drug, is not genotoxic but is non-selective as it kills normal dividing cells as well as cancer cells [5, 6].

Pancratistatin (PST) is a natural compound extracted from the *Hymenocallis littoralis* that has cyto-static anti-neoplastic (anti-tumourigenic growth) activity [7]. Recently, PST has been shown to selectively target cancer cells leading to apoptosis with minimal effect on non-cancerous cells [8]. PST has previously been reported to be effective against human neuroblastoma, leukemia and breast cancer cell lines [8, 9, 10]. It has been postulated that PST is non-genotoxic, since it bears no structural resemblance to any DNA intercalating drug or taxane and it does not cause double-stranded breaks or DNA nicks [7, 9].

Mitochondria are the most likely site of PST action; however, the mechanism of action and the protein target of PST in the mitochondria are still unknown [8].

PST in combination with Tamoxifen in ER(+) breast cancer cells have already shown a synergistic increase in apoptosis [10]. TAM is a drug for ER (+) advanced breast cancer adjuvant chemotherapy [11]. Estrogen encourages the growth of breast cancer cells. TAM competes with estrogen for the estrogen receptor, preventing it from binding and encouraging excessive growth. In turn, when TAM binds it induces apoptosis in ER (+) cells. More recently, TAM has been shown to target the mitochondria [12]. There is evidence to show that the synergy response, which occurs when PST is combined with TAM in an ER (-) breast cancer line, is possibly due to both drugs targeting the mitochondria [10].

Hyper-methylation of certain genes have been attributed to the chemo-resistant trait of human melanoma to chemotherapy. Such genes include multidrug resistance 1 (MDR1), death associated protein kinase 1 (DAPK1) and the estrogen receptor (ER)[13]. Levels of ER expression in melanoma have been under debate for sometime. Previous studies using monoclonal antibodies have indicated variability in the levels of α ER in melanoma [14, 15, 16, 17]. One study highlighted the fact that α ER is epigenetically silenced through hyper-methylation in melanoma [18]. The same group was the first to determine one of the mechanisms by which TAM treatment in melanoma would fail [17], thereby ruling out that strategy of treatment. However, mitochondrion being our proposed target makes our ideas to the mode of action of TAM contrary to the ER-directed mechanism. A recent study confirmed that as melanoma becomes invasive, there is a decrease in expression of ER α and ER β genes and the corresponding ER β protein [19]; decrease in ER β is now used as a marker for proliferative melanoma [19]. We therefore pose the question:

will this mitochondria-targeting combination of PST and TAM exhibit a synergistic induction of apoptosis in melanoma cells as it did in ER (-) breast cancer cells [10]?

In addition to this, we look at the effect of a compound similar to PST, narciclasine, on MCF-7 breast carcinoma. This cousin compound differs from PST by the loss of one hydroxyl group. The efficacy of pancratistatin (PST) in successfully inducing apoptosis in MCF-7 breast cancer cells was highlighted in a previous study performed by Siedlakowski and co-workers [10]. Furthermore, the estrogen receptor negative (ER-) counterpart of MCF-7, the Hs-578-T, also showed susceptibility to PST treatment. When PST was combined with tamoxifen, and increase synergistic induction of apoptosis was exhibited in both cell lines. As mentioned before, this indicated that tamoxifen could act independently of ER status and sensitize human breast carcinoma to the effects of PST treatment, and this combined effect was greater than that produced by either compound alone in both cell lines. Therefore, in a preliminary study we wanted to evaluate if narciclasine, a compound that differs by only one hydroxyl group, could perform in a similar, if not more effective manner than PST. Furthermore, we wanted to determine whether narciclasine could perform better than PST in combination with TAM.

In this chapter we present the efficacy of PST-induced apoptosis in human melanoma cells. We also indicate that apoptosis induced by PST is selective to cancerous cells only, leaving normal cells unharmed. Most importantly, we find that combination of PST and TAM leads to a greater induction of apoptosis in human melanoma cells than either compound alone. We illustrate that PST/TAM treatment

alone or in combination targets the mitochondria leading to apoptosis. This combination could provide a non-toxic treatment regime which could effectively and selectively kill melanoma cells. Furthermore in a subsequent study using TAM in combination with narciclasine (similar to PST structure), we show that narciclasine does induce apoptosis in MCF-7 cells on its own at doses similar to PST, however, in combination its effect with TAM is not as effective as PST and TAM in MCF-7 [10].

Materials and Methods

Cell Culture

Human melanoma cells (A375) were purchased from ATCC, Manassas, VA, USA. The cells were grown and cultured at 37 °C and 5% CO₂ in RPMI-1640 media (Sigma-Aldrich, Oakville, ON, Canada) containing L-glutamine and NaHCO₃ and was completed with 10% Fetal Bovine Serum, Canadian Origin (FBS) (Sigma, Canada) and 10µg/mL gentamycin (Gibco, Canada). In parallel to A375 human melanoma cells, experiments were conducted using G361 human melanoma cells (ATCC, Manassas, VA, USA). The cells were grown and cultured at 37 °C and 5% CO₂ in McCoy's Medium 5a (Gibco, Canada) modified with L-glutamine and completed with 10% FBS and 10µg/mL gentamycin.

MCF-7 breast cancer cells were also purchased from ATCC, Manassas, VA, USA. These cells were grown in Eagle's Minimum Essential Medium, completed with 10% FBS, (Sigma, Canada) and 10µg/mL gentamycin (Gibco, Canada) and 0.1% bovine insulin.

The Normal Human Fibroblast line (NHF) was purchased from the Coriell Institute for Medical Research, USA. They were grown at 37 °C and 5% CO₂ in Earle's minimum essential medium (Sigma-Aldrich, Oakville, ON, Canada) completed with 15% FBS, 10µg/mL gentamycin, 2mM L-glutamine and essential and non-essential amino acids and vitamins (Gibco, Canada).

Cell Treatment

Human Melanoma A375 cells were grown to 60-70% confluence and treated with Pancratistatin (PST) and/or Tamoxifen (TAM, tamoxifen citrate salt: Sigma-Aldrich, Oakville, ON, Canada) at the indicated concentrations and time points. These cells were also treated with methyl viologen dichloride hydrate (Sigma-Aldrich, Oakville, ON, Canada), which we will now refer to as paraquat (PQ). Estrogen receptor positive breast cancer cells, MCF-7s, were treated with narciclasine in the same manner, and in combination with TAM.

Apoptosis Assays

Cellular Staining

Following exposure to PST and/or TAM morphological features of apoptosis in A375 and NHFs cells were stained with 10 μ M final concentration Hoechst 33342 dye as per a previously published protocol [10]. Apoptotic cells have brightly stained, condensed nuclei compared to non-apoptotic cells. Cells were observed using a fluorescent microscope (Leica DM IRB); pictures were taken at 40X objective. The same protocol was applied to MCF-7 cells treated with narciclasine and tamoxifen.

Annexin-V Binding Assay

Annexin-V binding assay was performed following desired treatment and incubation periods using a purchased kit and manufacturer's protocol (Sigma-Aldrich, Oakville, ON, Canada). Briefly, cells were washed twice in PBS , re-

suspended in Annexin-V binding buffer (10mM HEPES, 10mM NaOH, 140mM NaCl, 1mM CaCl₂, pH 7.6), and incubated with Annexin-V conjugate (1:50) for 15 min at 25°C. Cells were observed using a fluorescent microscope (Leica DM IRB); pictures were taken at 40X objective. The same protocol was applied to MCF-7 cells treated with narciclasine.

WST-1 assay for cell viability

The cell viability assay was performed according to the manufacturer's protocol (Roche Applied Science, IN, USA) using the WST-1 reagent. Cell viability was measured as a function of active melanoma metabolism. Cell viability is high when the metabolic functions are unaffected. 96-well tissue culture plates were seeded with ~1000 cells/well and treated with the indicated concentrations of PST and TAM incubated for 18 hours. The WST-1 reagent was then added to each well and incubated for 4 hours at 37 °C, and then read at 450nm on a Wallac Victor³™ 1420 Multilabel Counter (Perkin Elmer™, Ontario, Canada). Absorbance readings were expressed as cell viability against percent control (cells with no treatment). The same assay was performed with MCF-7 cells that were treated, at the indicated concentrations of narciclasine and tamoxifen, in 96-well plates.

JC-1 Staining

Human melanoma cells grown on coverslips, were treated with PST and TAM for 72 hours. JC-1 dye was used to visualize collapse of the MMP according to a previously published protocol [10]. Briefly, JC-1 dye was added directly to the

culture media at 0.5 μ M final concentration and incubated for 1 h at 37°C. Cells were observed using a fluorescent microscope (Leica DM IRB); pictures were taken at 40X objective. The same protocol was carried out on MCF-7 cells treated with narciclasine and tamoxifen.

Mitochondrial Isolation and Amplex Red Assay

Intact mitochondria were isolated from untreated A375 cells based on previously published protocols [10, 20]. Briefly, cells were washed twice with ice-cold PBS and re-suspended in hypotonic buffer (1mM EDTA, 5mM TRIS-HCl, 210mM mannitol, 70mM sucrose). Cells were manually homogenized then centrifuged at 600 x *g* for 5 min at 4° C. The supernatant was centrifuged at 15000 x *g* for 15 min at 4° C. The resulting mitochondrial pellet was re-suspended in ice-cold reaction buffer (2.5mM malate, 10mM succinate in PBS). Isolated mitochondria were then directly treated with PST and/or TAM for the desired time and the level of reactive oxygen species (ROS) generated was measured with 5 μ M Amplex Red (Molecular Probes, Eugene, Oregon, USA). Equal volumes of mitochondrial isolate were added to an opaque 96-well plate with reaction buffer and the desired drug treatment(s), and were incubated together. The plate was read at Ex. 530nm and Em. 580nm on a spectrofluorometer (SpectraMax Gemini XS).

ROS Assay: Whole Cells

A375 cells were seeded on a 24-well clear bottom plate. At 60-70% confluence cells were treated in quadruplets with 1 μ M PST over a 24, 48 and 72 hour period. A 24 -hour treatment with 250 μ M of PQ was used as a positive control based on a previous finding; paraquat is known to induce high levels of ROS in cells and is therefore used as a positive control [21]. The fluorophore 2',7'-dichlorofluorescein diacetate (DCFDA) (Molecular Probes, Eugene, Oregon, USA) was added to each well (10 μ M final concentration) and incubated at 37°C for 15 min. The plate was then read on a spectrofluorometer (Spectra Max Gemini XS) at Ex. 495nm and Em. 530nm, every 3 minutes over 2 hrs at 37°C. Fluorescence was reported as RFU per 10⁶ cells; cells were counted using Trypan Blue exclusion dye (Sigma-Aldrich, Oakville, ON, Canada).

Caspase-3 Activity Assay

Caspase-3 activity assay was carried out as per a previously published method [9] and manufacturer's protocol (Enzyme System Products, USA). Briefly, A375 cell lysate was incubated for 1 h with the fluorogenic caspase-3 substrate (DEVD-AFC) at 37 °C in DEVD buffer. Fluorescence was measured at Ex. 400nm and Em. 505nm using a spectrofluorometer (Spectra Max Gemini XS). Caspase-3 activity was expressed per micrograms of protein. Protein concentration was measured by the Bradford Assay (BioRad) with BSA as a standard.

Results

Induction of Apoptosis by Pancratistatin in Human Melanoma Cells

Pancratistatin (PST), a natural compound, has been shown to successfully induce apoptosis in various cell lines including human neuroblastoma cells (SHSY-5Y), rat hepatoma (5123), human leukaemia (Jurkat) and human breast adenocarcinoma (MCF-7) [8, 9].

Melanoma is a very aggressive form of skin cancer for which effective chemotherapeutic treatment has been elusive. It is characterized for its notorious exhibition of chemoresistance to drugs. Until now PST had not been tested on melanoma.

Our first objective was to observe the efficacy of PST-induced apoptosis in the A375 human melanoma cell line. Cells were treated with PST at the effective concentration of 1 μ M previously determined by Pandey and co-workers, 2005 and at 0.5 μ M as described previously [6,8]. Hoechst dye was used to observe the extent of apoptosis induced after treatment. PST treatment at concentrations of 0.5 μ M and 1 μ M showed significant increase in apoptosis compared to control [Figure 1A]. Figure 1A and B are shown below (page 42) and the figure legend for Figure 1 is provided on page 43.

Figure 1

A

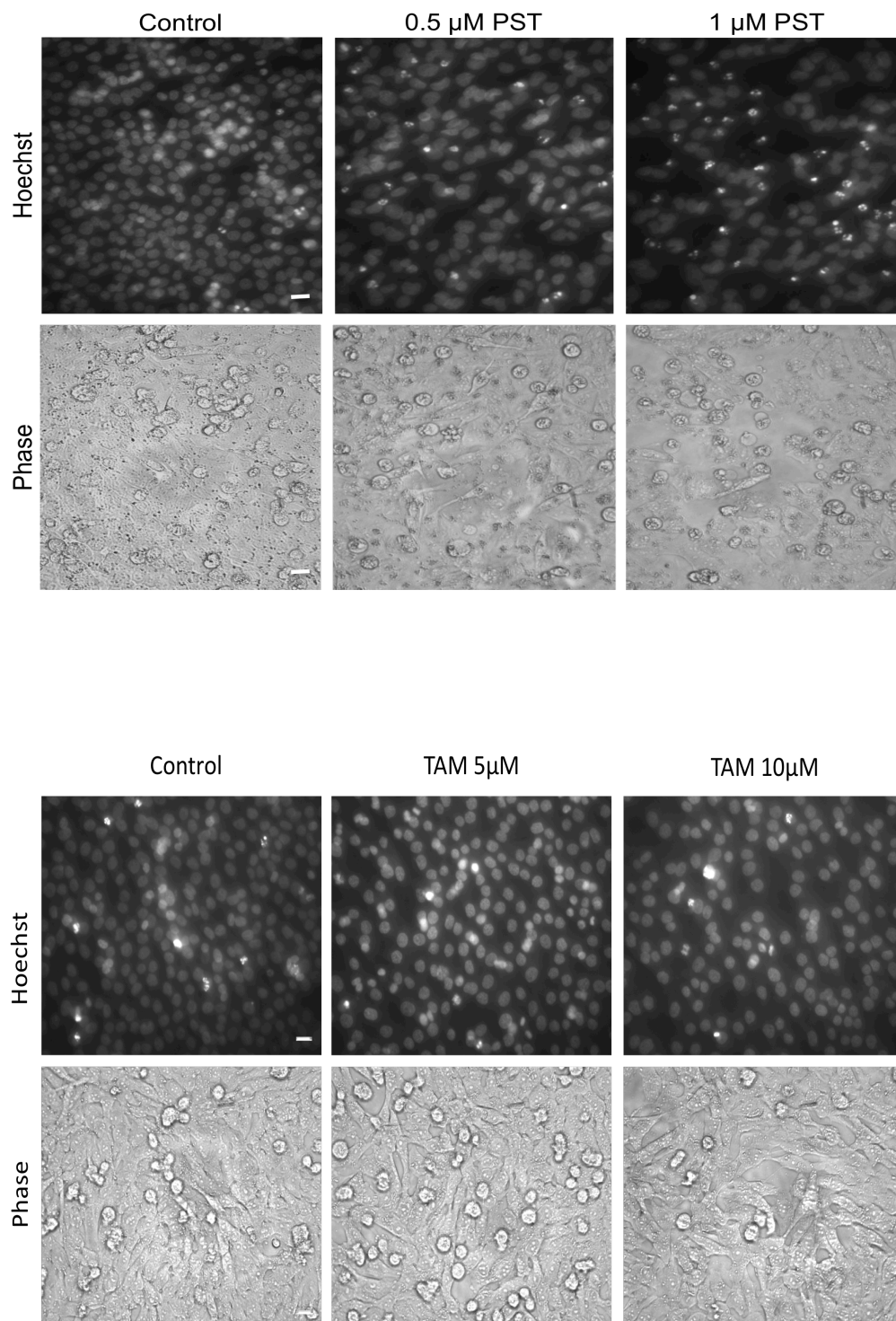


Figure 1: Hoechst images of A375 melanoma treated with PST and A375 melanoma treated with TAM for 72 hours. A) Human melanoma A375 cells were plated and treated with PST and B) TAM, as indicated in the figure, for 72 hours. Cells were then stained with Hoechst dye and were imaged using a fluorescent microscope as described in materials and method section. Scale bar for 1A and 1B is 15 microns as shown on the controls.

Synergistic Effect of Tamoxifen and PST in Inducing Apoptosis in A375 Cells

Tamoxifen (TAM) is a widely-used breast cancer drug that induces apoptosis by acting as an antagonist to the estrogen receptor of ER(+) breast cancer cells. Previous studies have shown that TAM may also act on the mitochondria to induce apoptosis [12, 22]. Recent studies have used a low concentration of TAM (5 μ M) in combination with another drug (lovastatin) in MCF-7s [23, 24]. Hoechst staining on A375 cells treated with TAM at both its effective concentration (10 μ M) and half-effective concentration (5 μ M) resulted in a very small increase in apoptosis compared to control [Figure 1B, page 43]. The effective concentration of TAM used was based upon a previous study [25], which indicated that TAM concentrations up to 1 μ M have a cytostatic effect in human breast carcinoma (MCF-7) cells; however, at 10 μ M TAM there is both cytostatic and cytolytic effects [25].

Our laboratory has recently reported that combination treatment of PST and TAM induces apoptosis synergistically in breast carcinoma [10]. Our results suggest that PST induces apoptosis at the mitochondrial level, and so to determine whether this synergistic response can solely occur through a mitochondria-dependent apoptotic pathway without involving receptor-mediated apoptosis, we tested TAM on A375 melanoma cells alone and in combination with PST. Though the mechanism of action of PST has not yet been elucidated, studies indicate that it targets the intrinsic pathway of apoptosis. Recently it was found that TAM also acts on the mitochondria via the flavomononucleotide site on complex I of the electron transport chain, resulting in destabilization of the mitochondria [12]. Considering

that the mitochondria may be a common target of both compounds, we wanted to examine whether the combination (PST and TAM) would produce a greater effect in melanoma cells at reduced concentrations to reduce toxicity to non-cancerous cells. An increase is observed in the number of apoptotic cells as the concentration of the co-treatment is increased from 0.5 μ M PST and 5 μ M TAM to 1 μ M PST in combination with 10 μ M TAM. At 72 hours, when half the effective dose of both drugs (0.5 μ M PST and 5 μ M TAM) is used in combination, there is approximately 40% apoptosis induced [Figure 2, page 46]. At the effective concentration of PST (1 μ M) and TAM (10 μ M) there is increased apoptosis (~53%) in combination-treated cells versus treatment with either 1 μ M PST or 10 μ M TAM alone, significant to the control.

Figure 2

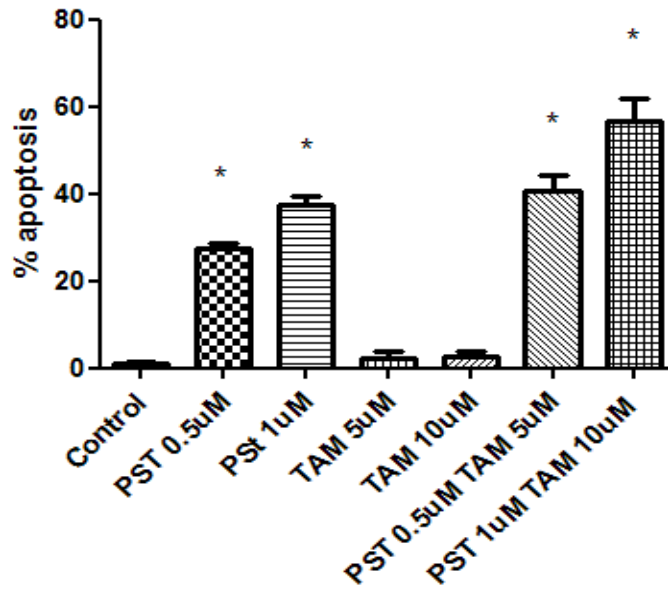
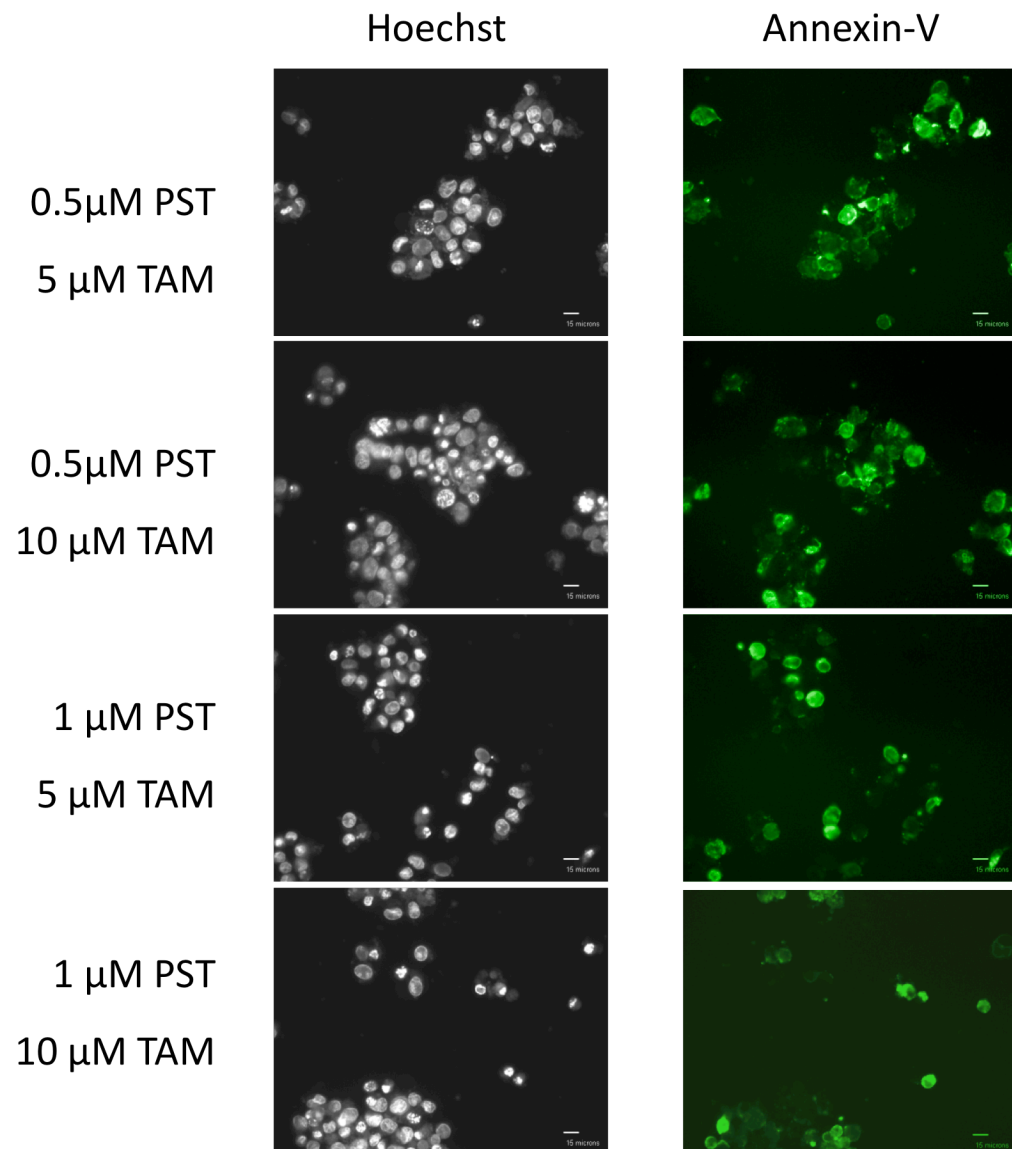


Figure 2: Quantification of PST and TAM co-treatment on A375 melanoma after 72 hours. Human melanoma A375 cells were treated with PST and TAM for 72 hours at the indicated concentrations and stained with Hoechst dye as described in Materials and Methods. Cells were imaged using a fluorescent microscope as described in Materials and Method section. Percentage of apoptotic cells were quantified using images from different fields (approx. 10 fields counted per treatment). Statistical analysis was performed using STATISTICA software; * denotes a p value <0.0005.

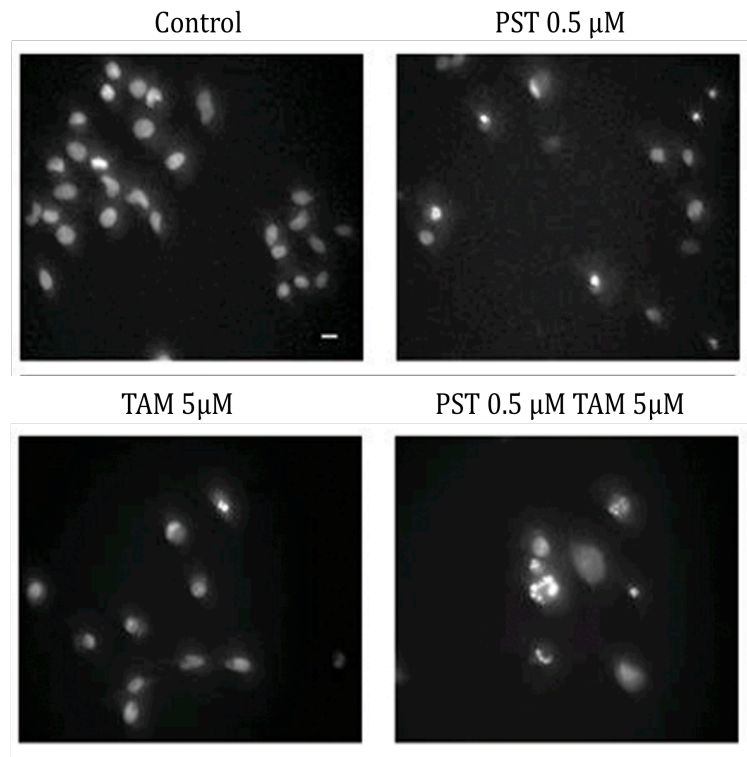
To confirm the results in combination the Annexin-V binding assay was performed. Flipping of phosphatidyl serine from the inner leaflet of the plasma membrane to the outer leaflet is an early event in apoptosis and is visible as an increase in fluorescence corresponding to an increase in binding of the Annexin-V FITC conjugate. Indeed, an increase in fluorescence was observed when the treatment concentration was increased from its lowest combinations of 0.5 μ M PST and 5 μ M TAM to 1 μ M PST and TAM 10 μ M [Figure 3A, page 48] (figure legend on page 50). The combinatorial treatment was then repeated with the G361 human melanoma cell line, and the effect was observed using Hoechst staining and Annexin V binding assay after 48 hours [Figure 3B and 3C, page 49]. G361 did appear to be responsive to PST and even the combination but were not as sensitive as the A375 cell line.

Figure 3

A



B



C

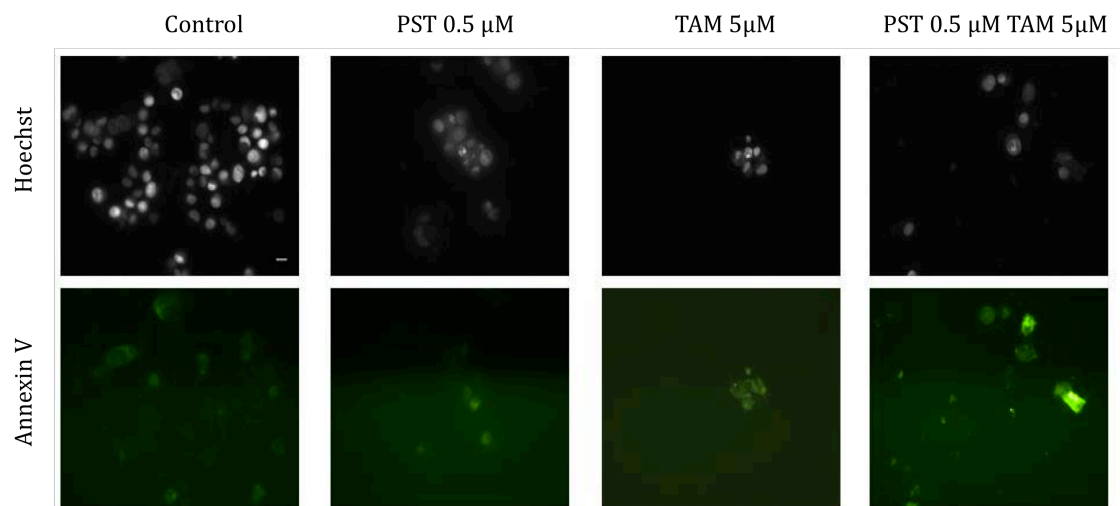


Figure 3: A) Effect of PST and TAM co-treatment on A375 cells after 72 hours. Human melanoma A375 cells were plated and co treated with PST and TAM (indicated above) for 72 hours, and stained with Annexin-V fluor and Hoechst dye as described in Materials and Methods. The treated cells were imaged using a fluorescent microscope described in materials and method section. Scale bar is 15 microns as shown in control. **B) Hoechst images of G361 melanoma cells treated with PST and TAM for 48 hours.** Cells were treated with 0.5 μ M PST and 5 μ M TAM and incubated for 48 hours. Hoechst dye was then added to the cells which were then imaged. Scale bar is 15 microns. **C) Confirmation of apoptosis induction by PST and TAM co-treatment on G361 cells after 48 hours.** G361 human melanoma cells treated for 48 hours with 0.5 μ M PST and 5 μ M TAM were stained with the Annexin-V fluor and Hoechst dye as described in Materials and Methods. The treated cells were imaged using a fluorescent microscope described in Materials and Methods. Scale bar is 15 microns.

Inhibition of A375 Cell Viability upon PST-TAM Treatment

Human melanoma A375 cells were treated with PST and/or TAM for 18 hours and observed for inhibition of cell viability using WST-1 reagent. After 18 hours the decrease in cell viability, based on melanoma cells' metabolic activity of mitochondrial dehydrogenases, was greater in combination of PST 0.5 μ M and TAM 5 μ M than for either treatment alone. Combination of PST 0.5 μ M with a TAM 5 μ M resulted in a small decrease from 40% with 0.5 μ M PST treatment to 20% in combination (0.5 μ M PST and 5 μ M TAM) [Figure 4A, on page 52] (figure legend for provided on page 52). The experiment was repeated with G361 human melanoma cells which responded well to PST treatment at 0.5 μ M, though were not as sensitive as the A375 cells [Figure 4B, on page 52].

Figure 4

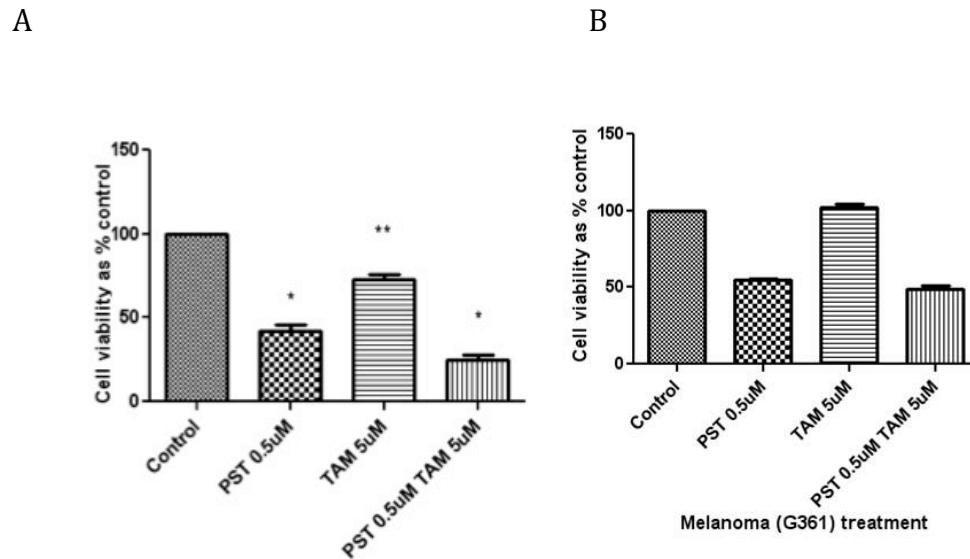


Figure 4: A) Measurement of A375 cell viability after PST-TAM co-treatment using WST-1. Melanoma A375s seeded in a 96 well tissue culture plate were treated at the indicated concentrations for 18 hours. After incubation with the WST-1 dye, as described in Materials and Methods, the wells absorbances were read at 450nm wavelength. Melanoma viability reduced in response to lowest combination PST and TAM treatments, than for either drug alone. Statistical analysis was performed using STATISTICA software; * denotes a p value < 0.001, ** denotes a p value < 0.005. **B) Measurement of G361 cell viability after PST-TAM co-treatment using WST-1.** G361 human melanoma cells were seeded at 1000cells/well in a 96 well plate. The cells were treated at the indicated concentrations and incubated for 18 hours. The WST-1 dye was added to each well and incubated for 4 hours as described in Materials and Methods.

Mitochondria as a Target of PST and TAM

Mitochondrial destabilization can be characterized by overproduction and accumulation of reactive oxygen species (ROS). Nearly all cellular ROS is derived from the mitochondria. To study the effect of PST and TAM treatment on mitochondrial destabilization, the levels of ROS generated by treatment were examined after 24, 48 and 72 hours. Paraquat (PQ), a toxic herbicide used in models of Parkinson's disease, was used as a positive control as it generates high levels of ROS [21]. The fluorophore used to detect ROS overproduction in this study is 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). Exposure of 1 μ M PST for 24 hours resulted in the greatest level of ROS generation compared to control [Figure 5, on page 54]. Figure 5 and its figure legend are provided on page 54.

Figure 5

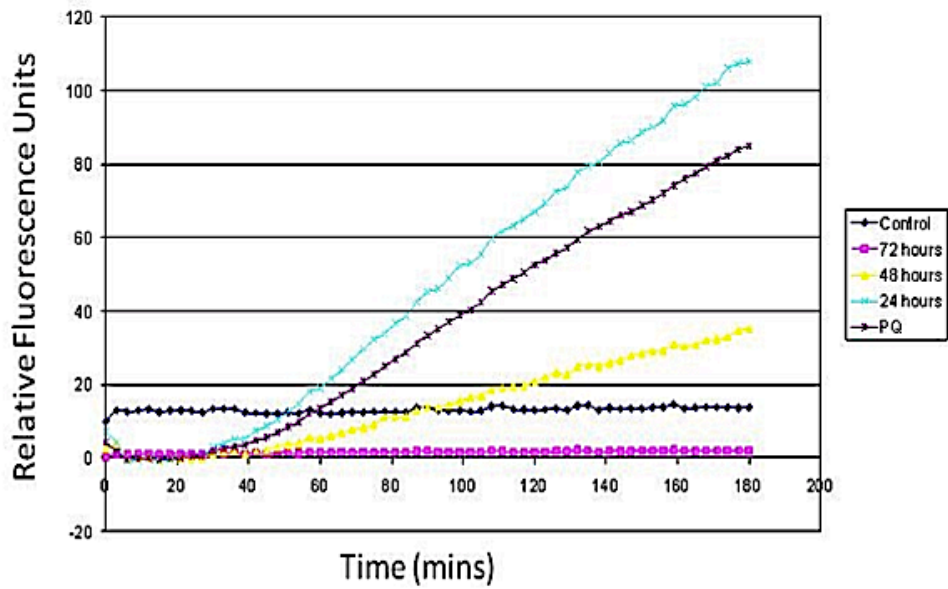


Figure 5: Total cellular ROS induced by PST in A375 cells. Human melanoma A375 cells were plated and treated with $1\mu\text{M}$ PST for 24, 48 and 72 hours or with paraquat (PQ) for 24 hrs. DCFDA was added to the cells after the treatments to quantify levels of ROS generation and fluorescence readings were taken over 3 hours following the end of each treatment period as described in Materials and Methods.

Depolarization of Mitochondrial Membrane following PST-TAM Treatment

Increased total cellular ROS production suggests the possibility of mitochondrial destabilization upon exposure to PST; to determine how PST and TAM affect the mitochondria together, mitochondrial membrane potential was assayed by JC-1 staining. Mitochondrial membrane potential collapse is a characteristic of apoptosis. Figure 6 (page 56) shows untreated (control) cells as having healthy mitochondria, in contrast to treatment with 0.5 μM PST and more so after treatment with 1 μM PST (data not shown). A375 cells treated with 5 μM TAM and 10 μM TAM (data not shown) did not show significant decrease in membrane potential. There was a slight decrease of JC-1 dye aggregation in cells exposed to 5 μM TAM compared to control, but a more pronounced decrease in cells treated in combination with 5 μM TAM and 0.5 μM PST compared to control and the singular treatments.

Figure 6

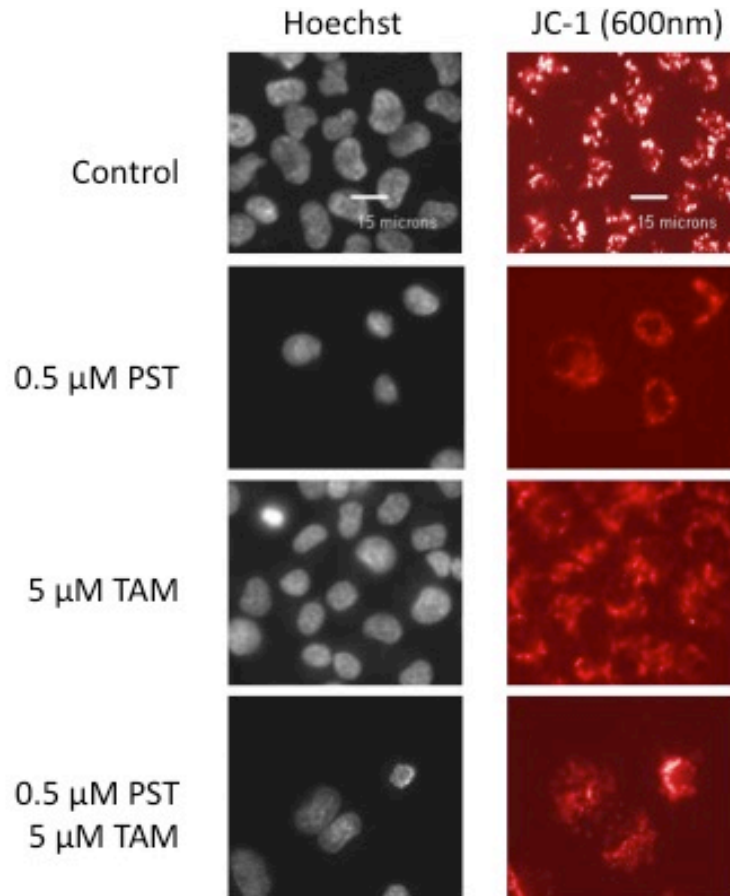


Figure 6: Loss of mitochondrial membrane potential following PST-TAM combination treatment in A375 cells. Human melanoma A375 cells were treated either with PST or TAM or both for 72 hours at the indicated concentrations and stained with JC-1 dye and imaged using a fluorescent microscope (described in materials and methods). Red fluorescence indicates intact mitochondrial membrane potential (MMP) and disappearance of red fluorescence indicates collapse of MMP. Scale bar is 15 microns as shown in the control figures.

Mitochondrial Reactive Oxygen Species

Upon observing total ROS generation in whole melanoma cells after PST and TAM exposure, we then examined the effect of the compounds on isolated mitochondria of A375 cells. Isolated mitochondria were treated with 0.5 μ M PST and/or 5 μ M TAM to determine the extent of ROS generation, indicative of apoptosis, and possible synergy of combined treatment at these low concentrations. The co-treatment of 0.5 μ M PST and 5 μ M TAM results in a greater increase in the level of ROS generated per microgram (μ g) of mitochondrial protein than for either treatment alone [Figure 7]. This effect may be attributed to both drugs targeting the mitochondria.

Figure 7

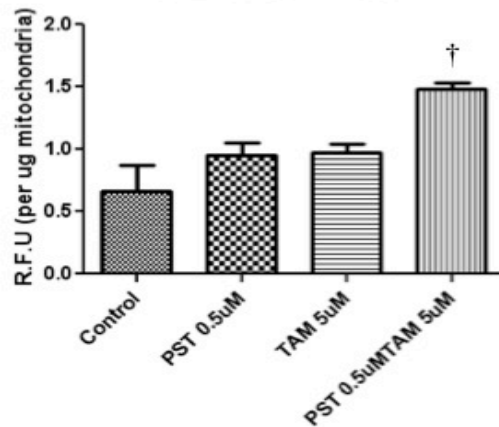


Figure 7: Mitochondrial ROS for PST-TAM co-treatment at lowest concentrations in A375 cells. Mitochondria were isolated from human melanoma A375 cells, and directly treated with PST and TAM as indicated above. Amplex Red was used to measure levels of ROS in presence of horseradish peroxidase as described in Materials and Methods. All treatments indicate destabilization of the mitochondria compared to the control, with a significant synergistic increase in the combined treatment; † denotes $p < 0.005$.

Caspase 3 Assay for PST-TAM Combination Treatment

Caspase-3 is an executioner caspase that is activated in the later stages of apoptosis [26]. To determine if caspase-3 gets activated after a 48 hour exposure to PST-TAM combination (0.5 μ M and 5 μ M, respectively), a caspase-3 activation assay was performed. Figure 8 shows significant activation of caspase-3 for all indicated treatments compared to untreated (control) cells.

Figure 8

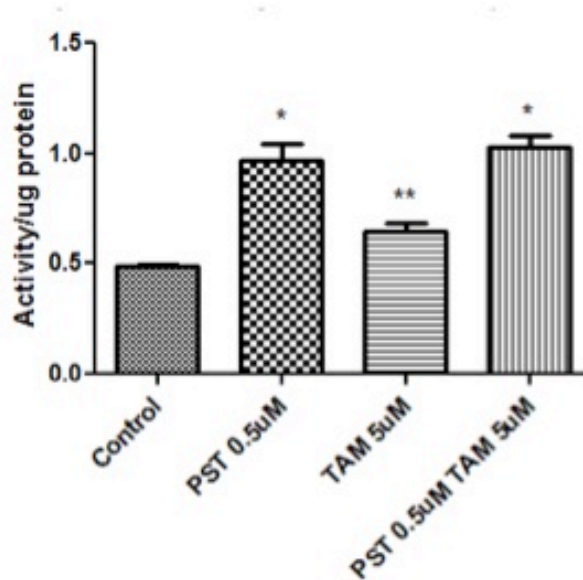


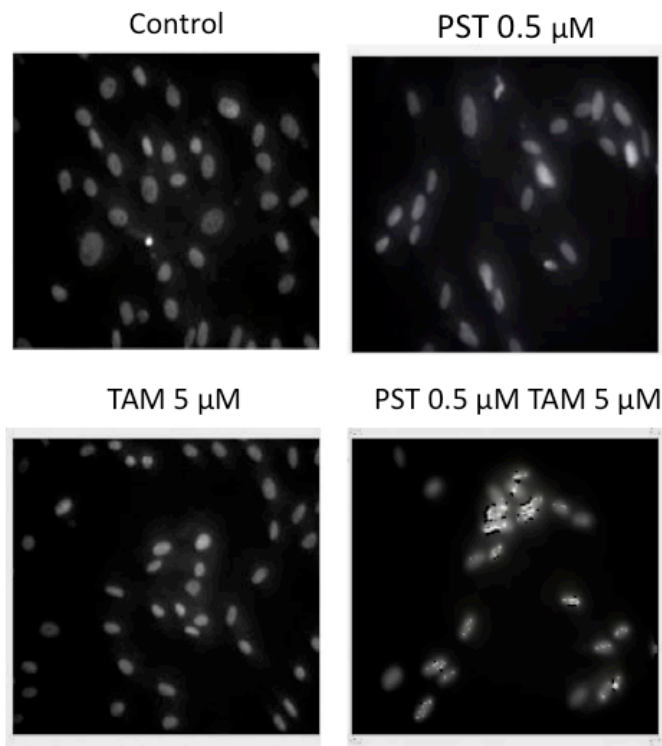
Figure 8: Caspase-3 activation upon PST and TAM co-treatment after 48 hours in A375 cells. Human melanoma A375 cells were treated with PST and TAM for 48 hours as indicated. Caspase-3 activity was measured as described in Materials and Methods. A significant increase in the level of caspase-3 activation is observed in all treatments compared to the control. Statistical analysis was performed using STATISTICA; * indicates a p value < 0.005; ** indicates a p value < 0.05.

Normal Cells Remain Unaffected by High Concentration PST-TAM Treatment

Normal Human Fibroblasts (NHFs) were used as a non-cancerous cell counterpart to melanoma cells to examine possible toxic effects of combination treatment of PST and TAM. NHFs were treated with 1 μ M PST, 10 μ M TAM, or 1 μ M PST and 10 μ M TAM for 96 hours. We show that there was minimal effect on the cells as observed by their nuclear morphology with Hoechst staining [Figure 9A and 9B] (Figure 9B along with the legend for Figure 9 are on page 60). It has been previously reported that there is no effect on mitochondrial membrane potential or ROS levels in NHFs treated with 1 μ M PST for up to 96 hrs [8].

Figure 9

A



B

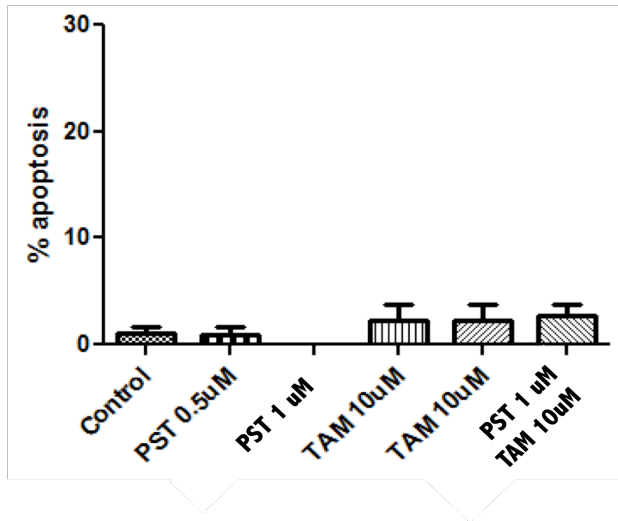


Figure 9: Hoechst staining and quantification for the effect of PST-TAM and the combined treatment on NHFs after 96 hours. A) Observing the effect of PST-TAM on NHFs using Hoechst. Normal Human Fibroblasts were grown to 60% confluence and treated with PST and TAM as indicated above for 96 hours. Hoechst dye was added after the treatment and cells were imaged using a fluorescence microscope. **B) Quantification of apoptotic cells by manual counting.** Quantification of cells from the images shows no change in apoptotic induction between the indicated treatments.

Long-term Effect on Melanoma Cells after initial exposure to PST-TAM Treatment

Considering that melanoma is generally more chemoresistant than most other carcinomas, we wanted to determine whether A375 cells would demonstrate resilience *in vitro* after an initial exposure of 1 μ M PST and 10 μ M TAM combination treatment. After initial exposure of PST-TAM (96 h), we wanted to determine if the remaining percentage of non-apoptotic cells are capable of growing again if the drugs are removed. Following the initial exposure of 96 hour treatment, the media was removed and these cells were given fresh drug-free media and monitored for an additional 96 hours. Our data suggests that these cells remain sensitive to PST mono-treatment and combinatorial treatment and have retained the signal to commit suicide after 96 hours, as the number of viable cells greatly decreased [Figure 10, page 62].

Figure 10

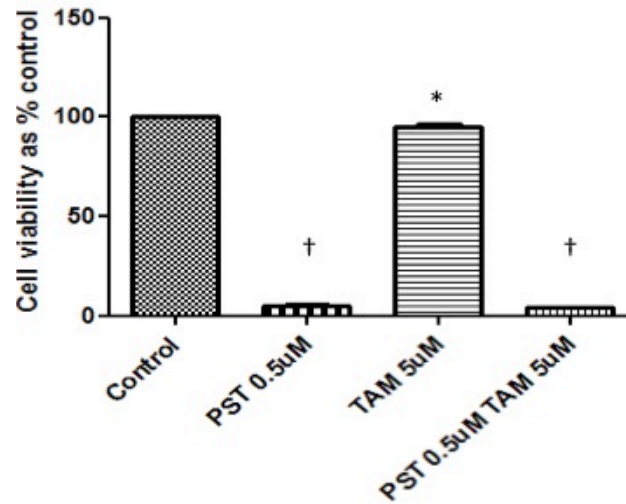


Figure 10: Apoptosis continues upon removal of drugs from PST-TAM treated A375 melanoma. Human melanoma A375 cells are treated with 1 μ M PST and 10 μ M TAM for 96 hours. Following this treatment period media was replaced without PST or TAM and cells were allowed to recover for another 96 hours. Cells were stained with Hoechst dye and counted as described in materials and methods. Melanoma A375 cells remain sensitized to the effects of initial combination treatment. Statistical analysis was performed using STATISTICA; * indicates a p value < 0.01; † indicates a p < 0.0005.

Effect of narciclasine and tamoxifen on MCF-7 breast cancer cells

MCF-7 breast cancer cells were treated using narciclasine at various concentrations for 72 hours and stained with Hoechst 33342 dye. Brightly stained nuclei start to appear after 0.5 μM narciclasine, and most appear dead after 1 μM narciclasine treatment [Figure 11]. There is a distinct increase in apoptotic nuclei as the concentration is increased from 0.5 μM narciclasine to 0.75 μM narciclasine. Apoptosis was confirmed at these concentrations using the Annexin-V binding assay [Figure 12, on page 64]. Hoechst staining also revealed that tamoxifen at 5 μM showed no effect on MCF-7 cell viability, however 10 μM TAM did [Figure 11] and this result correlates with previous findings by Siedlakowski and co-workers [10].

Figure 11

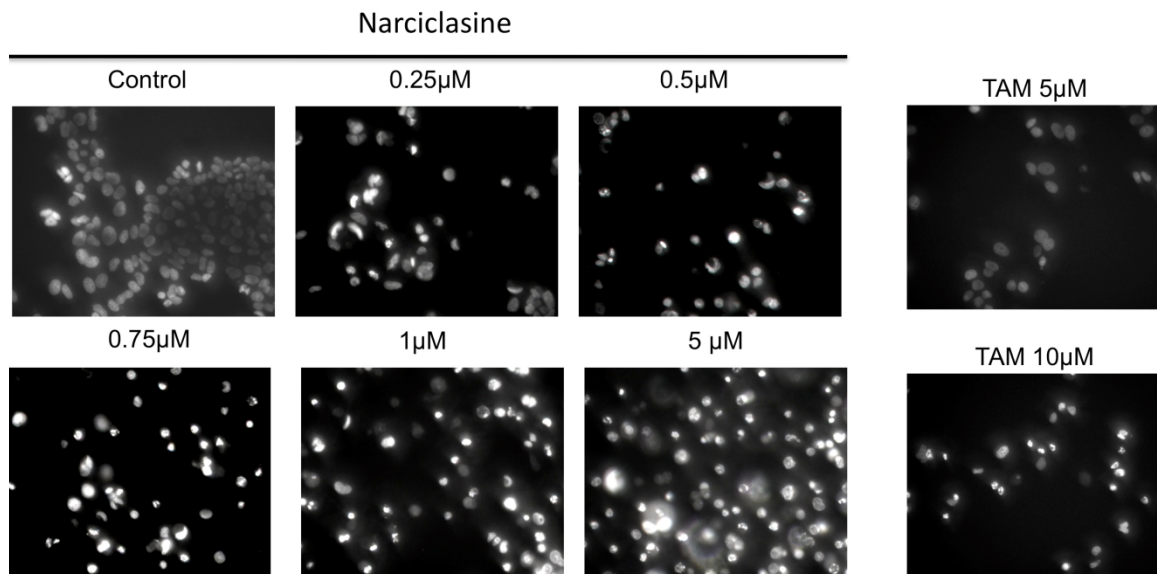


Figure 11: Hoechst images of MCF-7 cells treated with narciclasine and tamoxifen (separately) 72 hours. MCF-7 were plated and treated with narciclasine and TAM, as indicated in the figure, for 72 hours. Cells were then stained with Hoechst dye and were imaged using a fluorescent microscope as described in materials and method section.

Figure 12

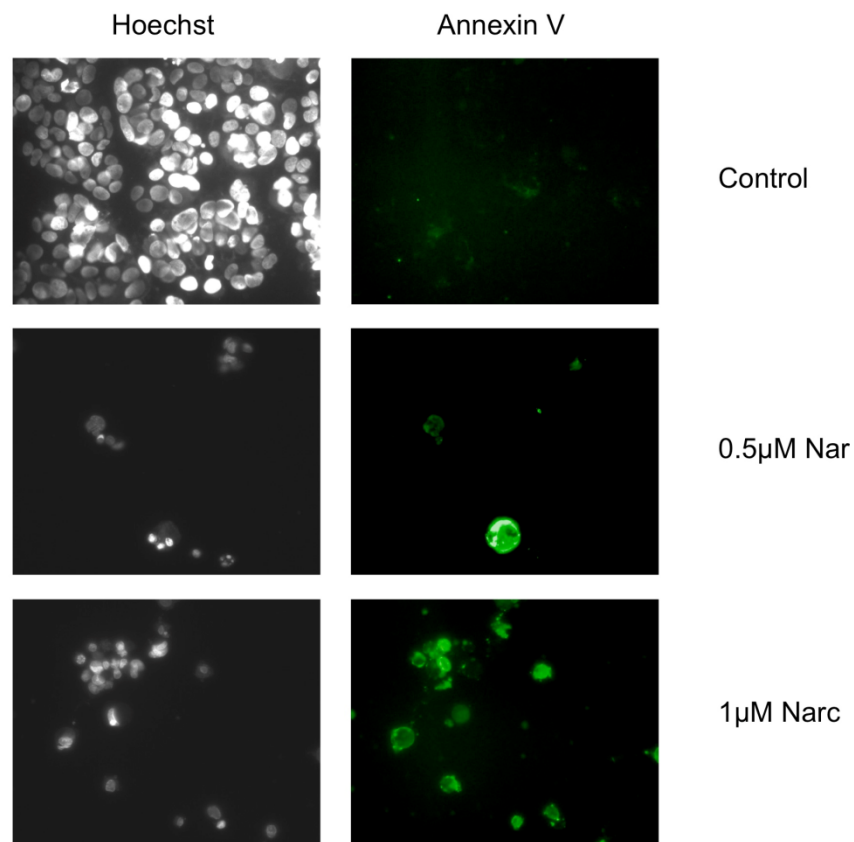
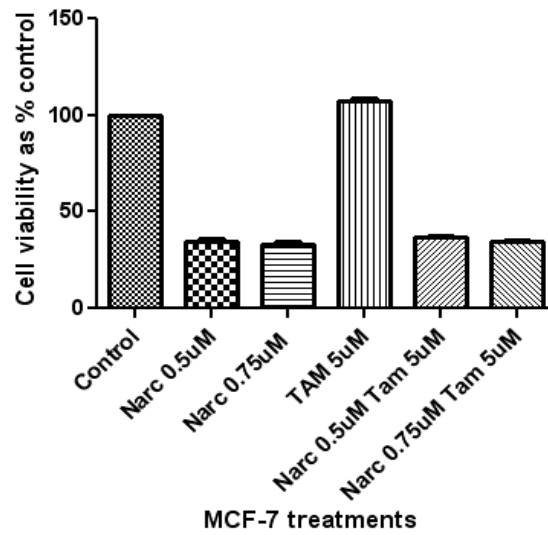


Figure 12: Confirmation of apoptosis after 72 hours narciclasine treatment on MCF-7 cells. MCF-7 cells treated for 72 hours with 0.5μM narciclasine and 1 μM narciclasine were stained with the Annexin-V AlexaFluor and Hoechst dye as described in Materials and Methods. The treated cells were imaged using a fluorescent microscope described in Materials and Methods.

Using the WST-1 assay narciclasine shows a 50% reduction in cell viability within 24 hours (data not shown) and by 72 hours there is approximately 30% viability at 0.5 μM [Figure 13a, page 66] (legend for Figure 13 is given on page 67). However, in combination with 5 μM TAM, there proved to be no additive or synergistic decrease in cell viability. To further determine if there could be any synergistic effect at all, further studies were completed using a combination of 0.5 μM narciclasine with 10 μM TAM. Dumont and co-workers also showed that narciclasine completely dissipates the mitochondrial membrane potential in MCF-7 breast carcinoma by 24 hours of treatment at 1 μM narciclasine. Narciclasine treatment at 0.1 μM was also found to be very effective [27, 28]. Figure 13B shows that at 0.5 μM of narciclasine there is still detectable levels of JC-1 staining compared to control [Figure 13B, page 66]. TAM treatment results in a slight disappearance of punctate marks compared to the punctate marks that appear in healthy mitochondria of the control. The combination using 0.5 μM narciclasine, though it does not show a decrease in cell viability with 5 μM TAM, does show greater mitochondrial membrane potential dissipation with 10 μM TAM than either compound alone.

Figure 13

A



B

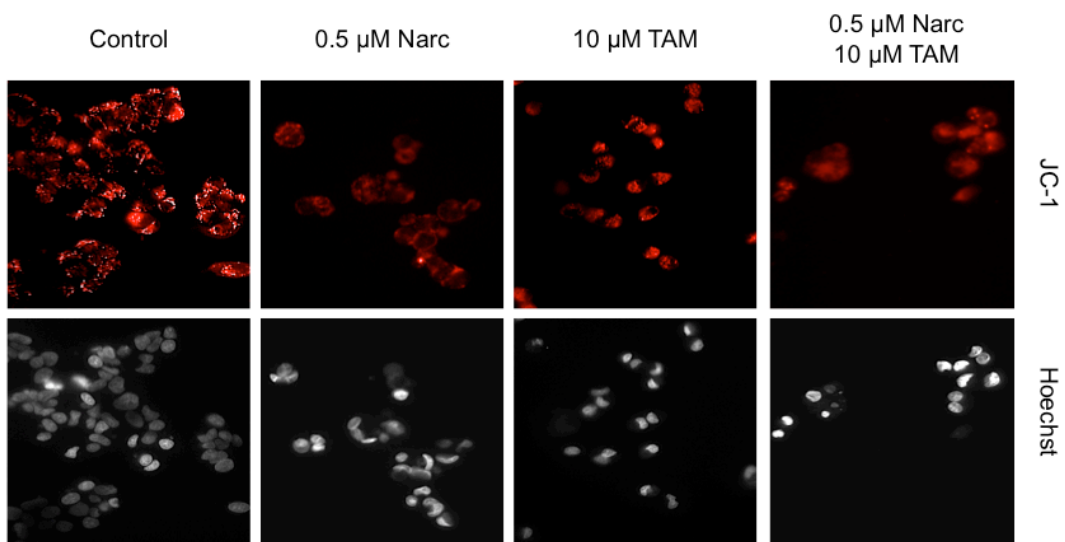


Figure 13: The effect of narciclasine and tamoxifen on MCF-7 mitochondria. A) Cell viability of MCF-7s post narciclasine and tamoxifen treatment measured by WST-1. MCF-7 cells were seeded in a 96 well tissue culture plate and were treated at the indicated concentrations for 72 hours. After incubation with the WST-1 dye, as described in Materials and Methods, the wells absorbances were read at 450 nm wavelength. **B) Loss of mitochondrial membrane potential (MMP) following narciclasine-TAM combination treatment in MCF-7 cells.** MCF-7 were treated either with narciclasine and/or TAM for 72 hours at the indicated concentrations. Cells were stained with JC-1 dye and imaged using a fluorescent microscope (described in materials and methods). Red fluorescence indicates intact mitochondrial membrane potential (MMP) and disappearance of red fluorescence indicates collapse of MMP.

Discussion

Inducing apoptosis in A375 melanoma cells

The aim of chemotherapy and radiation is to decrease the rate of cell division by targeting the DNA of cancer cells; apoptosis ensues, albeit non-specifically in non-cancerous cells as well. In this report we demonstrate that Pancratistatin (PST) and Tamoxifen (TAM) work together to induce apoptosis in A375 human melanoma cells.

Melanoma is an aggressive and potentially fatal form of skin cancer that currently does not have an effective treatment option. PST has been shown to induce apoptosis in a variety of cancer cell lines [6, 8, 9, 10]; however, the effect of PST on melanoma is being studied here for the first time. We report that melanoma is sensitive to treatment with 1 μ M PST, as apoptosis is induced within 72 hours. TAM is used to treat ER(+) breast cancer patients and our data suggests that TAM has no effect when used alone against A375 cells. Importantly, we show that combination treatment of PST and TAM (0.5 μ M PST and 5 μ M TAM, and also 1 μ M PST and 10 μ M TAM) induces apoptosis in A375 cells more effectively than either PST or TAM alone. This effect was also observed in another human melanoma cell line (G361) treated with PST and TAM at their lowest concentrations [Figure 4B]. Furthermore, experimental evidence indicates that the mitochondria are being targeted by PST and TAM. It could be possible that, although TAM is not actively inducing apoptosis in these cells, it may be sensitizing the mitochondria to the effects of PST exposure.

PST is a non-genotoxic compound that does not resemble any current DNA-targeting anti-cancer drugs. Unlike other natural compounds with anti-cancer potency such as Paclitaxel, which is also toxic to normal cells, PST does not significantly affect non-cancerous cells. It is hypothesized that PST acts on the mitochondria leading to caspase-dependent apoptosis possibly targeting a specific protein(s) that is critical to survival of cancer cells but relatively underused in normal cells. This hypothesis may explain how PST induces apoptosis specifically in cancer cells.

Recent studies have shown that TAM can induce apoptosis via a mitochondria-dependent mechanism, possibly binding to and destabilising the flavomononucleotide site on complex I in the electron transport chain [12]. Indeed, in MCF-7 (ER(+)) breast cancer cells it was demonstrated that PST treatment combined with TAM induced apoptosis synergistically, and more importantly, it was observed that PST and TAM worked together to induce apoptosis in ER(-) breast cancer cells [10]. Previous work has also shown that, at 1 μ M, PST does not influence normal cell replication and at low concentrations does not coerce cancer cells to become resistant [10]. We now report an observed increase in apoptosis induced by combined treatment with PST and TAM (1 μ M and 10 μ M, respectively) over the effect achieved with either compound alone. This effect may be attributed to both drugs acting on the mitochondria, in a manner independent of ER status. PST and TAM are both non-toxic compounds that have minimal effect on normal, non-cancerous NHF cells, and the synergistic effect against cancerous A375 cells is a most significant finding.

Targeting the Mitochondria with PST-TAM

Since mitochondrial destabilization and apoptosis are intimately linked and cancer is mainly glycolysis-driven with respect to metabolic processes (by-passing oxidative phosphorylation [29]), mitochondria could be considered vulnerable targets for development of cancer selective drugs. ROS over-production in cancer cells caused by chemotherapeutic drugs is indicative of mitochondrial destabilization, and leads to oxidation of mitochondrial membrane lipids, mitochondrial proteins, mitochondrial DNA and nuclear DNA [30-34].

Whole cell ROS levels measured after treatment with PST at 1 μ M at 24, 48 and 72 hours indicate that mitochondrial destabilization in A375 cells occurs as a result of excessive ROS generation [Figure 5]. Interestingly, exposure to 1 μ M PST for 24 hours produces the highest level of ROS generation over any other time point. At 72 hours, ROS production was lowest due to very low cell viability determined by the Trypan Blue exclusion assay (data not shown), and the level remained constant due to defunct mitochondria.

To investigate if these compounds directly destabilize the mitochondrial membrane, we studied the effect of PST and TAM (0.5 μ M and 5 μ M, respectively) on isolated mitochondria. Measurement of ROS generated by isolated mitochondria upon direct treatment with PST and TAM exhibited that this combination generates elevated levels of ROS than either PST or TAM alone. Although we did see TAM cause a substantial increase in mitochondrial ROS, the same concentration is unable to induce apoptosis in A375 human melanoma cells, indicating that TAM sensitizes

mitochondria to the effect of PST without completely destabilizing it. Previous studies have determined depolarization of the mitochondrial membrane potential upon treatment of cancer cells using a JC-1 cationic dye [10, 35, 36]. JC-1 staining was also carried out to show that there was depolarization of the mitochondrial membrane potential upon combination treatment [Figure 6]. Treatment of A375 cells with PST-TAM (0.5 μ M and 5 μ M, respectively) combination lead to the greatest collapse of mitochondrial membrane potential over either treatment alone.

To observe A375 cell viability in response to PST and/or TAM treatment, cells incubated with WST-1 dye produced a soluble formazan product metabolized by, and reflecting the overall activity of, mitochondrial dehydrogenases. Cell viability being a function of metabolic activity in this assay, is seen to decrease more with the combined treatment of PST and TAM at 0.5 μ M and 5 μ M, respectively, than either treatment alone [Figure 4]. A similar effect is seen in G361 human melanoma cells [Figure 4B].

Response of A375 cells after PST-TAM treatment.

In this study, we aimed to determine whether combined treatment of PST and TAM was capable of inducing cell death in all cells of a given population long after the initial exposure. Results show that melanoma cells responded well to the primary 96 hour treatment, and up to 96 hours afterwards these cells continued to commit suicide even when drugs were removed and media was replaced with fresh

drug-free media, rather than thrive in the absence of treatments as expected of a chemoresistant cell line [Figure 10].

Pan-caspase inhibitors have been used in blocking caspase-dependent apoptosis pathways. We carried out an experiment with Z-VAD-FMK (10 μ M) to see if it could inhibit A375 cell death by PST and in combination with TAM. Surprisingly, treatment with just the minimal concentration of the inhibitor on its own resulted in toxicity (data not shown). Therefore, it was impossible to conclude whether caspases are important for PST-TAM induced apoptosis. If mitochondria are destabilized by this dual PST-TAM treatment, then there may be other pro-apoptotic proteins (such as AIF and Smac/DIABLO) working independently of caspases, further inciting the subcellular target as being mitochondria.

Currently, the mechanism of action of PST remains under investigation. Evidence to the mode of action of PST in A375 cells include the activation of caspase-3, mitochondrial membrane potential collapse and destabilization of the mitochondria membrane. The combination of PST-TAM (0.5 μ M and 5 μ M, respectively) causes apoptosis via direct destabilization of cancer cell mitochondria, thereby exploiting an underlying vulnerability of cancer cells. This combination presents a possible novel, non-genotoxic treatment for melanoma. Our current research focus is to determine the cellular target of PST and if this target protein is uniquely modified in cancer cells, which would explain the observed selective apoptosis-inducing capability of PST in a broad range of cancer cell types. We propose that PST treatment is sensitized by combination with TAM, and may be the

answer to treatment of melanoma, a widely chemoresistant cancer. Further testing of this synergistic effect in an *in vivo* model of melanoma would be critical for the development of this novel therapeutic strategy.

Effect of Narciclasine and Tamoxifen on MCF-7 Breast Cancer

In a similar preliminary study, the effect of the compound narciclasine on MCF-7 breast carcinoma was determined in combination with TAM [10]. In a study conducted by Dumont and co-workers, narciclasine effectively reduced average cancer viability to 50% at nano-molar concentrations ($\sim 30\text{nM}$); however, studies with narciclasine were later performed using $1\text{ }\mu\text{M}$ instead [27]. MCF-7s showed delayed DNA laddering at 72 hours compared to the other cell lines tested using $1\text{ }\mu\text{M}$ narciclasine. This agrees with our observation whereby MCF-7 cells started to appear as apoptotic bodies at $0.5\text{ }\mu\text{M}$, and this effect was predominant at $1\text{ }\mu\text{M}$ narciclasine. Narciclasine-induced apoptosis was then confirmed using Annexin-V binding assay, which clearly showed major phosphatidyl serine flipping at $1\text{ }\mu\text{M}$. Based on these observations we pursued the combination of narciclasine with tamoxifen. The WST-1 assay indicated that at $0.5\text{ }\mu\text{M}$ and $0.75\text{ }\mu\text{M}$ concentrations of narciclasine, though there was a considerable reduction in cell viability, in combination with $5\text{ }\mu\text{M}$ TAM (non-cytotoxic to MCF-7), there was no further decrease in cell viability. However, when placed in combination with $10\text{ }\mu\text{M}$ TAM, narciclasine started to show greater effect in mitochondrial membrane potential dissipation. Therefore, the combination of $0.5\text{ }\mu\text{M}$ narciclasine and $5\text{ }\mu\text{M}$ TAM

showed no promise, as pancratistatin did at 0.5 μ M, it shows some effect at 10 μ M TAM. Though not understood why, we find that the loss of one hydroxyl group, reduces the effectiveness of a compound in a combinatorial treatment.

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Chapter 3

*The Efficacy of Dandelion Root Extract in Inducing Apoptosis in
Drug-resistant Human Melanoma Cells*

Abstract

Notoriously chemo-resistant melanoma has become the most prevalent form of cancer North American young adults. Standard treatment after early detection involves surgical excision (recurrence is possible), and metastatic melanoma is refractory to immuno-, radio- and most harmful chemotherapies. Various natural compounds have shown efficacy in killing different cancers, albeit not always specifically. In this study we show that dandelion root extract (DRE) specifically and effectively induces apoptosis (2.5 mg/mL) in human melanoma cells without inducing toxicity in non-cancerous cells. Characteristic apoptotic morphology of nuclear condensation and phosphatidyl serine flipping to the outer leaflet of the plasma membrane of A375 human melanoma cells was observed within 48 hours. DRE-induced apoptosis activates caspase-8 in A375 cells early on, demonstrating employment of an extrinsic apoptotic pathway to kill A375 cells. Reactive Oxygen Species (ROS) generated from DRE-treated isolated mitochondria indicates that natural compounds in DRE can also directly target mitochondria. Interestingly, the relatively resistant G361 human melanoma cell line responded to DRE when combined with the metabolism interfering anti-type II diabetic drug metformin (4mM). Therefore, treatment with this common, yet potent extract of natural compounds has proven novel in specifically inducing apoptosis in chemo-resistant melanoma, without toxicity to healthy cells.

Introduction

Melanoma skin cancer is among one of the leading cancers targeting adolescents and young adults in the North America. Melanoma is notoriously chemoresistant and the modes of treatment for melanoma are very limited, relying mainly on surgical excision of the primary site during early detection, and whatever limited chemotherapy and immunotherapy for metastasized melanoma that is available. However, these therapies have limited success and incur side effects [1].

Taraxacum Officinale is most commonly known as dandelion. Regarded as a regular garden weed, this detoxifying herb has long been used in traditional Chinese medicine, for ailments ranging from digestive disorders to complex disorders such as uterine, breast and lung tumours [2]. Traditional Middle Eastern remedies require dandelions for spleen and liver ailments, while Native Americans have harnessed their properties to cure indigestion, heartburn and kidney disease [2, 3]. Dandelion plants were (*Taraxacum officinale* Weber ex Wiggers) were used by East Indians in the 16 century as a hepatic stimulant, diuretic, for liver disorders, and most interestingly, for chronic skin diseases [4, 5]. These roots are a source of triterpenes and steroids [4, 6]. Hata *et al.*, found that upon screening a variety of compounds from wild plants, *Taraxacum Officinale* was an effective inducer of differentiation in mouse melanoma cells. Furthermore, this group found that one constituent of Chinese dandelion, Lupeol-a triterpene, up-regulated melanogenesis and decreased cell proliferation in mouse melanoma [7]. This triterpene is regarded as cytostatic (stops cell growth) and not cytotoxic (harmful to cells). In another

study, skin tumours were promoted *in vivo* in mice through a two-stage chemical carcinogenesis, and treated with water and methanol extracts of *Taraxacum japonicum*. This showed inhibition of tumour initiation and promotion at both carcinogenesis stages, and it was concluded that Taraxacum, and more specifically taraxasterol (a triterpenoid), is a worthy chemopreventative agent [8, 9].

More recently, Jeon *et al.*, have shown that ethanolic *Taraxacum Officinale* extracts and derivative forms thereof, reduce levels of reactive oxygen species (ROS), nitric oxide production (NO) and inhibit COX-2 expression or its anti-oxidant activity, thus making dandelion extracts not only anti-carcinogenic, but anti-inflammatory, anti-angiogenic and also anti-nociceptive [10]. Dandelion flower extracts (DFE) were tested on RAW264.7 cells (mouse macrophages) and exhibited inhibition of NO production in these non-cancerous cells. The inhibition of reactive nitrogen species (RNS) as well as ROS by DFE was attributed to its phenolic components [11]. Sigstedt *et al.*, tested aqueous DFE, dandelion leaf extract (DLE) and dandelion root extract (DRE), from *Taraxacum Officinale*, on a variant MCF-7 breast cancer cell line (MCF-7/AZ) and LNCaP prostate cancer line. While DLE inhibited cell proliferation in MCF-7/AZ, DFE and DRE did not. However, DRE and DLE blocked invasion of MCF-7/AZ and LNCaP cells (into collagen type I), respectively. Inhibition of cell invasion was corroborated by reduced matrix metalloproteinase activity of MMP-2 and -9, as well as reduced phosphorylation levels of src and FAK [2]. *Taraxacum Officinale* extract was used to treat Hep G2 human hepatoma cells and was found to reduce cell viability and induce cytotoxicity through interleukin- α and TNF- α [12]. Regardless of the valuable traditional

knowledge of *Taraxacum* anti-tumor activity, there has been inadequate biochemical research to apply this knowledge to cancer cell lines and especially chemo-resistant melanoma.

Population studies have highlighted the correlation between diabetic patients taking Metformin and a reduced incidence of cancer and lower rates of mortality. Jiralerspong *et al*, had clinically proven this by determining that diabetics with breast cancer taking Metformin, had a higher pathological complete response than diabetic breast cancer patients not taking the drug [13]. Normally, healthy cells express adenosine monophosphate-activated protein kinase (AMPK), which is directly downstream of the tumour suppressor LKB1, and is usually activated as a reaction to cellular energy stress. Metformin can also activate AMPK, leading to suppression of the ATP consuming pathways (e.g. gluconeogenesis) and activation of the ATP generating pathways (e.g. glycolysis). Though the mechanisms for metformin treatment of cancer are not clearly understood, it is clear that cancer cell energy metabolism differs from that of normal cells, and this difference makes tumourigenic cells vulnerable to metabolism interfering drugs like metformin [14].

In this study, we have investigated the effect of dandelion root extract on human melanoma cell lines *in vitro*. For melanoma, a very aggressive, chemo-resistant form of skin cancer, we have shown that DRE has been very effective in inducing apoptosis. We have also seen that the extract targets the mitochondria, generating reactive oxygen species. It is possible that the compounds in the extract work through the extrinsic apoptotic pathway, as is indicated by elevated levels of early caspase-8 activation. We have also observed that drug-resistant melanoma

cells could be made more sensitive to DRE treatment by the metabolism interfering drug, metformin. This is the first time that a study has been performed with metformin and human melanoma. Though it is unclear which components of DRE are active in successfully killing human melanoma cells, our work with *Taraxacum* DRE presents a novel, natural chemotherapeutic agent that maybe extended to other chemo-resistant cancer lines.

Materials and Methods

Cell Culture

Human melanoma cell lines, A375 and G361, were purchased from ATCC (Manassas, VA, USA). A375 human melanoma cells were grown and cultured at 37 °C and 5% CO₂ in RPMI-1640 media containing L-glutamine and NaHCO₃ (Sigma-Aldrich, Oakville, ON, Canada) completed with 10% Fetal Bovine Serum (FBS) (Sigma, Canada) and 10µg/mL gentamycin (Gibco, Canada). G361 human melanoma cells were cultured in McCoy's Medium 5a modified with L-glutamine (Gibco, Canada) and completed and grown in the same manner as the A375s.

Normal human Fibroblasts (NHF) were purchased from the Coriell Institute for Medical Research, USA. NHFs were cultured in Earle's Minimum Essential Medium (Sigma-Aldrich, Oakville, ON, Canada) completed with 15% FBS, 2mM L-glutamine, 10µg/mL gentamycin, vitamins, and essential and non-essential amino acids (Gibco, Canada).

Cell Treatment

Water extracts of dandelion root were prepared using variety of filtering stages, lyophilized, constituted to a particular stock concentration, and sterilized. Indicated concentrations of 2.5mg/mL translate to 125µg of DRE that the cells are exposed to. Similarly, 1mg/mL, 5mg/mL and 10mg/mL correspond to 50µg, 250µg

and 500µg of DRE, respectively. Cells (A375 and G361 human melanoma cells, and NHFs) were treated with DRE at a 60% confluence, at the indicated concentrations and time points. G361 cells were treated with metformin (1,1-dimethylbiguanide hydrochloride- Aldrich, USA) at a concentration of 4mM. This concentration was based upon work done by previous groups, which had used concentrations ranging from 0.5 to 10 mM metformin to treat human breast carcinoma cells [15]; Isakovic *et al* used concentrations upto to 8 mM to treat glioma cells [16], and concentrations of metformin reached as high as 20 mM to inhibit mTOR in human breast carcinoma [14].

Cell Viability

Cell viability was measured using a WST-1 reagent (Roche Applied Science, IN, USA). A375s (~1000 cells), G361s (~3000 cells) and NHFs (~ 2000 cells) were plated with a fixed number of cells per well 96-well tissue culture plates. After 24 hours the cells were treated with DRE at the indicated concentrations in triplicate. WST-1 reagent was added to the wells after the treatment period, and incubated at 37°C for 4 hours. The plates were then read at 450 nm on a Wallac Victor³™ 1420 Multilabel Counter (Perkin Elmer™, Ontario, Canada). Absorbance readings are expressed in terms of cell viability as percent control (untreated cells).

Cellular Staining

Cells treated with DRE for the specified time periods were stained with Hoechst 33342 dye (final concentration 10 μ M) as per a previously published protocol [17], to image the cellular morphological features. Apoptotic cells are characterized by condensed, brightly stained nuclei. Non-apoptotic cells are not condensed or brightly stained. Cells were imaged with the dye using a fluorescent microscope (Leica DM IRB); pictures were taken at 40X objective.

Annexin-V Binding Assay

To confirm DRE-induced apoptosis in, Annexin-V binding assay was performed after 48 hours with DRE using a purchased kit and the manufacturer's protocol (Sigma-Aldrich, Oakville, ON, Canada). Post treatment cells were washed twice with PBS and re-suspended in Annexin-V binding buffer (10mM HEPES, 10mM NaOH, 140mM NaCl, 1mM CaCl₂, pH 7.6). Annexin-V-FITC conjugate (1:50) was added to the cells and incubated for 15 minutes at room temperature. Cells were observed and imaged under a fluorescent microscope (Leica DM IRB); images were taken at 40X objective.

Caspase-8 Activity Assay

Caspase-8 activity was assayed using the manufacturer's procedure (Enzyme System Products, USA). Cell lysates were prepared using A375 human melanoma cells and incubated for 1 hour with caspase-8 fluorogenic substrate (IETD-AFC) in IETD buffer at 37°C (Calbiochem) in 96-well opaque microtitre plates. AFC fluorescence was measured at 400nm excitation and 505nm emission using spectrofluorometer (SpectraMax Gemini XS). Caspase activity was measured expressed in terms of relative fluorescence units per µg protein, and protein concentration was determined using Bradford Assay (BioRad); Bovine Serum Albumin (BSA) was used as a standard.

Observation of Mitochondrial Membrane Potential Depolarization

Human melanoma A375 cells were seeded on sterile coverslips in 6-well tissue culture plates, and treated with DRE for 24 hours. After DRE treatment, the cells were incubated for 1 hour with JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzamidazolocarbocyanin iodide) at a final concentration of 0.5µM at 37°C. Cells were observed and imaged using a fluorescent microscope (Leica DM IRB); images were taken at 40X objective.

Measurement of Mitochondrial Reactive Oxygen Species

Mitochondria were isolated from A375 cells based on a previously published protocol [18]. The mitochondria were treated directly with DRE in 96-well opaque microtitre plates. Amplex Red (5 μ M) and horseradish peroxidase (HRP) were added to the mitochondria post-treatment. The fluorescent resorufin product was measured at 530nm excitation and 580nm emission using spectrofluorometer (SpectraMax Gemini XS). Relative Fluorescence Units were measured and expressed in terms of per μ g protein, and protein concentration was determined using Bradford Assay (BioRad); Bovine Serum Albumin (BSA) was used as a standard.

Post-Treatment Cell Revival

A375 cells pre-treated with DRE for 72 hours, were trypsinized and re-plated in drug free RPMI1640 media, and incubated for 96 hours at 37 °C and 5% CO₂. Cells were stained using Hoechst 33342 dye and imaged using a fluorescent microscope (Leica DM IRB); pictures were taken at 40X objective. Cells were also collected and counted using Trypan blue exclusion assay.

Results

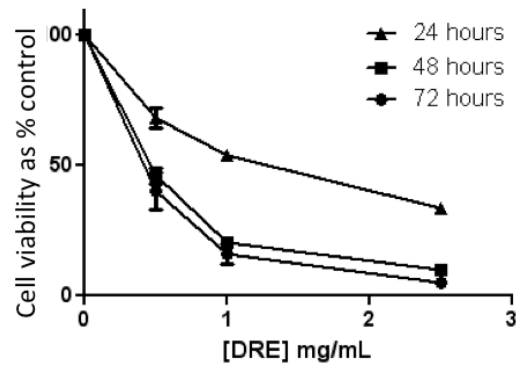
Effect of Dandelion Root Extract (DRE) on human melanoma cell viability

In order to investigate whether Dandelion Root Extract (DRE) reduced cell viability in human melanoma cells, the A375 cells were treated with 1, 2.5 and 5mg/mL concentrations of DRE. DRE was found to reduce cell viability in a dose-dependent fashion, over time, in A375 melanoma cells as was measured by WST-1 assay. Based on metabolic activity of A375s it was confirmed that treatment at 2.5mg/mL DRE resulted in ~ 50% reduction in cell viability against control within 24 hours [Figure 1A, page 91] (figure legend for Figure 1 is provided on page 92). After cells were imaged with Hoechst dye and it was found that by 48 hours there was a clear induction of apoptosis at concentrations above 2.5mg/mL [Figure 1B, page 91], as distinguished by brightly stained nuclei and the morphological features of condensation and fragmentation.

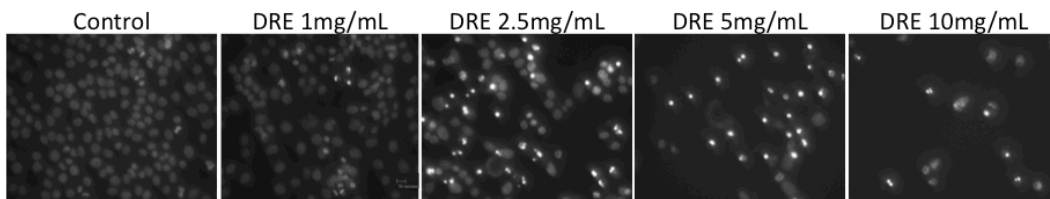
Using the effective and sub-effective doses, we sought to confirm that apoptosis in A375 cells was indeed induced, using the Annexin-V binding assay. The assay confirms that by 48 hours the phosphatidyl serine has flipped from the inner leaflet of the plasma membrane to the outer leaflet after treatment with 2.5mg/mL DRE [Figure 1C, page 91].

Figure 1

A



B



C

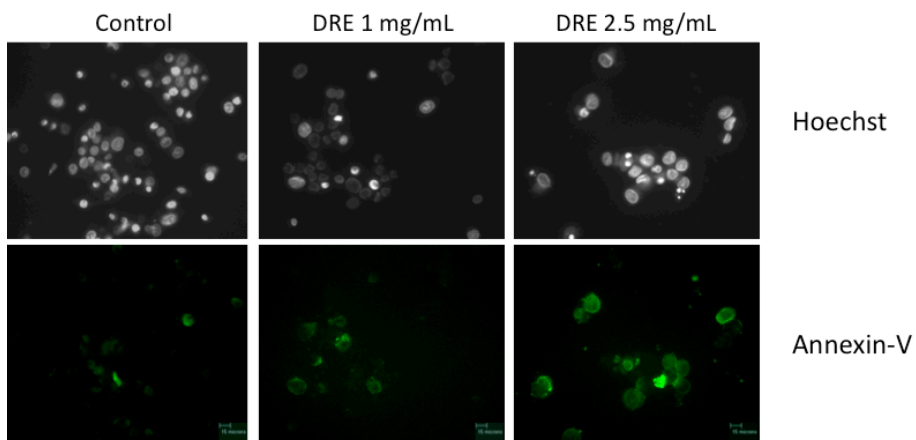


Figure 1: Determining the sensitivity of A375 human melanoma cells to DRE.

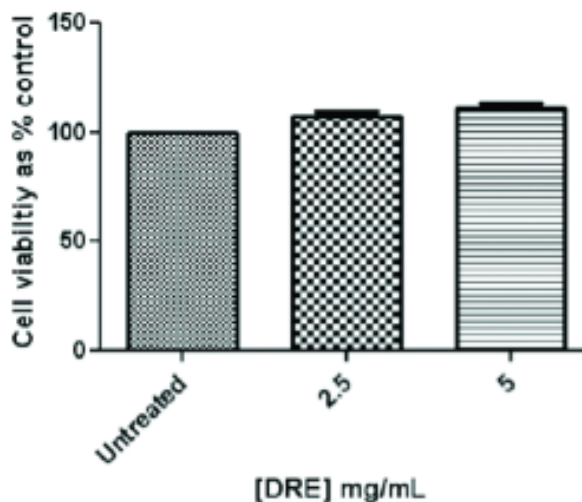
A) Effect of DRE on cell viability. A375 human melanoma cells were seeded on 96-well plates (~1000cells/well) and treated at the indicated concentrations for 24, 48 and 72 hours. The WST-1 dye was added to each well after every treatment period and incubated, as described in Materials and Methods. Absorbances were read at 450nm. **B) Induction of apoptosis by DRE after 48 hours.** Typical apoptotic morphology was observed in A375 cells treated with DRE (0-10mg/mL concentrations) for 48 hours. Cells were stained with Hoechst 33342 dye, before images were taken on a fluorescence microscope. Brightly stained, condensed bodies indicate apoptotic nuclei. **C) Confirmation of apoptosis by Annexin-V binding assay after 48 hours.** Cells treated at the effective and sub-effective dose of 2.5 and 1 mg/mL respectively for 48 hours, were stained with Annexin-V Alexa Fluor (green) following which cells were imaged on a fluorescence microscope.

Evaluation of DRE toxicity on Normal Human Fibroblasts

With DRE having proven its efficacy in successfully killing this aggressive, chemoresistant form of skin cancer, DRE toxicity on normal cells had to be evaluated. Taraxacum extracts have been used for centuries and therefore have it is assumed that normal cells are unaffected by it. Regardless, Normal Human Fibroblasts (NHF) were used as a normal counterpart to human melanoma cells to test this assumption. DRE was treated at the effective and sub-effective doses of DRE (1 and 2.5mg/mL). After a long exposure of 96 hours, NHFs did not exhibit any reduction in cell viability [Figure 2A] (legend for Figure 2 provided on page 94), and this was further supported by the lack of brightly stained, condensed and fragmented nuclei (characteristic of apoptosis) when stained with Hoechst dye [Figure 2B, page 94].

Figure 2

A



B

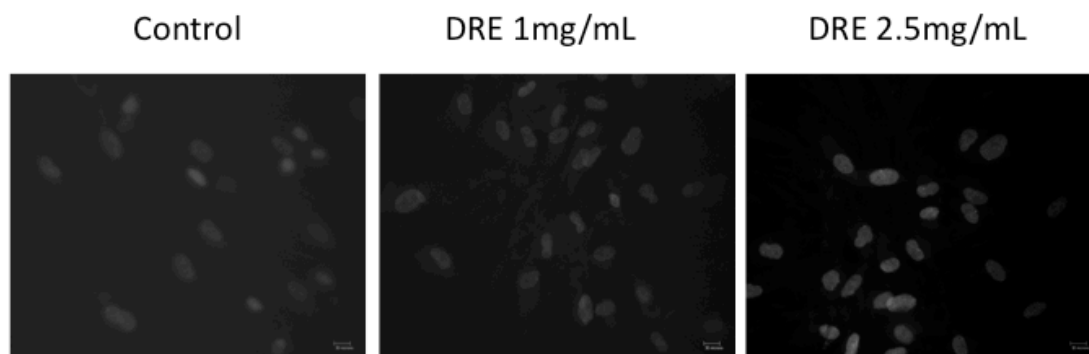


Figure 2: Determining the toxicity of DRE on Normal Human Fibroblasts (NHF). A) Effect of DRE on NHF cell viability after 96 hours. NHFs were seeded on a 96- well plate (~2000cells/well) and treated with DRE at the indicated concentrations for 96 hours. The WST-1 dye was added to each well after the 96-hour treatment period and incubated as described in Materials and Methods. Absorbances were read at 450nm. B) **Non-toxic effect of DRE on NHFs by Hoechst staining.** NHFs were treated with DRE at the indicated concentrations for 96 hours. Cells were stained with Hoechst 33342 dye and imaged on a fluorescence microscope, as described in Materials and Methods.

Analysis of cell death pathways employed by DRE

To determine how apoptosis is being induced in A375 cells, we wanted to assess whether there was cell surface death-receptor mediated induction would result in caspase-8 activation. Incubation of the A375 whole cell lysate with the IETD-AFC substrate revealed that the caspase- 8 was activated and that the substrate was cleaved within 30 minutes of DRE treatment [Figure 3].

Figure 3

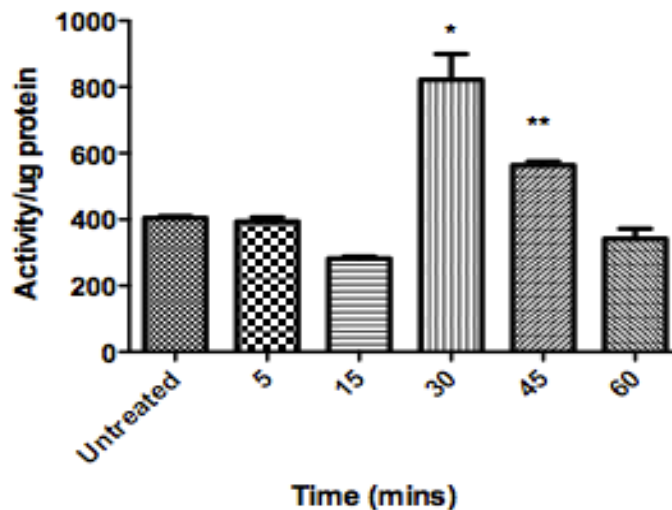


Figure 3: Caspase-8 activity in DRE treated A375 cells. A375 cells were treated at 2.5mg/mL for the indicated periods of time. Cells were collected and incubated with caspase-8 IETD-AFC substrate for an hour (as per the protocol) before being read on a spectrofluorometer as described in Materials and Methods. Statistical analysis was performed using the GraphPad Prism 5.0, * denotes a p-value <0.05; ** denotes a p-value <0.01.

Considering early activation of a death receptor mediated pathway, we wanted to then assess the effect of DRE on the mitochondria by observing polarization/depolarization across the mitochondrial membrane using the JC-1 dye. The JC-1 dye aggregates if the mitochondrial membrane potential is maintained, as shown by red punctate marks. By 24 hours, the effective dose of 2.5mg/mL DRE indicated the dissipation in the mitochondrial membrane potential, indicated by fewer punctate marks, as a result of the dye's inability to aggregate in the mitochondria with lost potential [Figure 4].

Figure 4

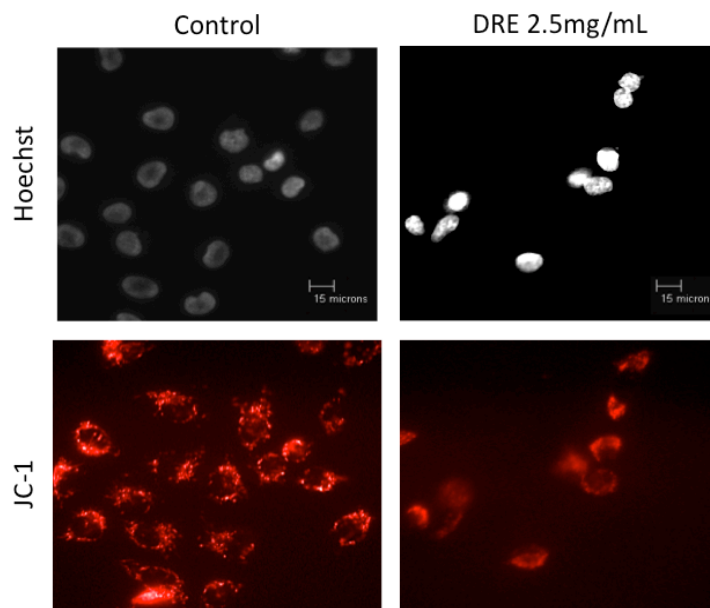


Figure 4: Dissipation of the mitochondrial membrane potential in A375 cells. A375 cells were seeded on coverslips and treated with DRE for 24 hours. The JC-1 was then added and cells were incubated for 1 hour, before being imaged on a fluorescent microscope, as described in Materials and Methods. Punctate marks indicate healthy mitochondria.

In order to determine whether the ROS generation in A375 melanoma cells was as a direct result of DRE or not, mitochondria isolated from the A375 melanoma cells were treated with DRE at 1 and 2.5mg/mL concentrations. Mitochondrial ROS was measured over 25 minutes for 2-minute intervals. A steady increase in the levels of mitochondrial ROS was observed compared to untreated control. At 0 minutes (T=0) there is a 2.5mg/mL DRE generated higher levels of reactive oxygen species than 1mg/mL (data not shown). However, over time both concentrations showed the same rate of ROS production compared to control [Figure 5].

Figure 5

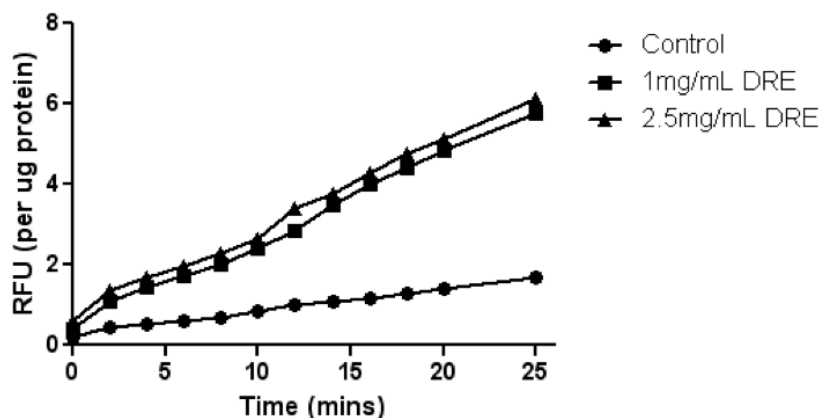


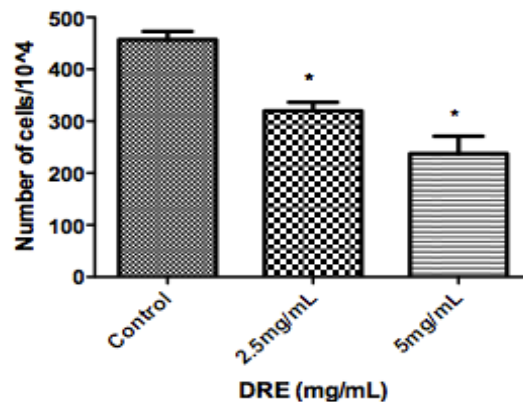
Figure 5: Assessing levels of DRE-induced ROS generation in A375 mitochondria. Mitochondria were isolated from A375 cells as described in Materials and Methods, and treated with DRE. Amplex Red and HRP were added and fluorescence was measured on the SpectraMax Gemini XS.

Evaluating A375 melanoma cell revival post-treatment

To ascertain the long-term efficacy of DRE on chemo-resistant melanoma, A375 cells were treated with at 2.5 and 5mg/mL for 72 hours before being replated in drug-free media, and incubated for 96 hours. The Trypan blue count after 96 hours shows a reduction in cell viability upon increasing concentration. The viable cells counted also account for membrane un-permeabilized apoptotic cells [Figure 6A] (the legend for Figure 9 is provided on page 99), and these apoptotic cells can be observed by Hoechst staining [Figure 6B, page 99]. In Figure 6B, the apoptotic nuclei can be clearly distinguished by the brightly stained nuclei.

Figure 6

A



B

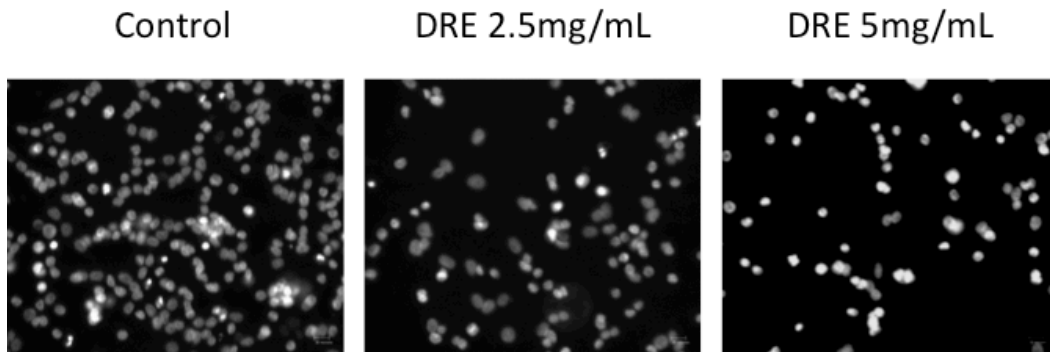


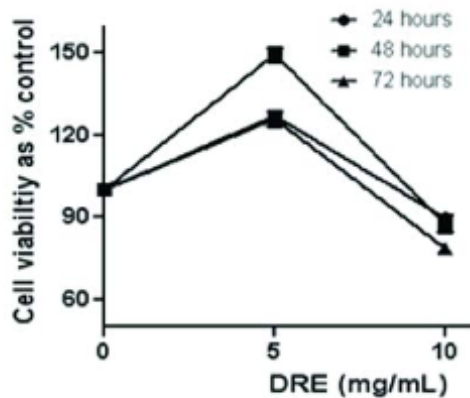
Figure 6: Quantification and observation of A375 cell-revival (96 hours) post 72 hours DRE-treatment. A) Quantification of A375 cells after drug-free incubation (revival). Negatively stained A375 cell count post DRE-treatment. A375 cells were treated with DRE, at the indicated concentrations, for 72 hours, following which they were re-plated in fresh drug-media and incubated for 96 hours as described in Materials and Methods. Negatively stained cells were collected and counted using Trypan blue. Statistical analysis was performed using the GraphPad Prism 5.0, * denotes a p-value <0.05. **B) A375 Hoechst images after revival.** Continued apoptosis induction in A375 cells post DRE-treatment. A375 cells were treated with DRE at the indicated concentrations for 72 hours, as described in Materials and Methods. After 96 hours incubation in drug-free media cells were stained with Hoechst 33342 dye and imaged on a fluorescence microscope.

Effect of DRE on G361 human melanoma cells

To further determine the effect of DRE on other melanomas, we used the G361 human melanoma cell line and treated these cells for 72 hours at the same concentrations of DRE as the A375s. These doses proved to be ineffective in reducing cell viability and inducing apoptosis in G361 cells (data not shown). Higher doses were then used and a response was observed at a concentration of 10mg/mL [Figure 7A and B]. Figure 7B and the legend for figure 7 are provided on page 101.

Figure 7

A



B

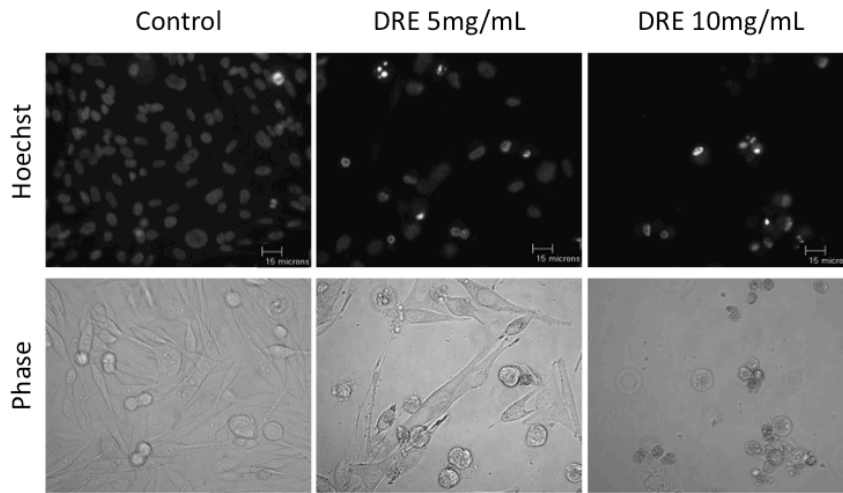
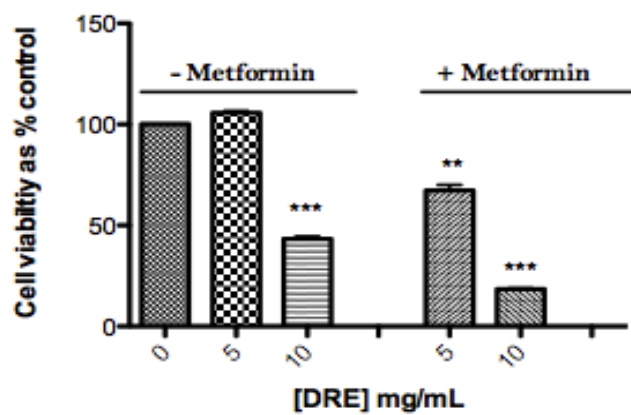


Figure 7: Effect of DRE on G361 human melanoma cells after 72 hours. A) Effect of DRE on G361 cell viability. G361 human melanoma cells were seeded on 96-well plates (~3000cells/well) and treated at the indicated concentrations for 72 hours. The WST-1 dye was added to each well after every treatment period and incubated, as described in Materials and Methods. Absorbances were read at 450nm. **B) Induction of apoptosis in G361 cells by DRE.** Typical apoptotic morphology was observed in G361 cells treated with DRE at 10mg/mL concentrations for 72 hours. Cells were stained with Hoechst 33342 dye, before images were taken on a fluorescence microscope. Brightly stained, condensed bodies indicate apoptotic nuclei.

In combination with the anti-type II diabetes drug and metabolism interfering drug, metformin, these relatively more resistant melanoma cells were sensitized to the effects of DRE even at lower doses of 5mg/mL [Figure 8A and B]. The corresponding figure legend for Figure 8 is provided on page 103.

Figure 8

A



B

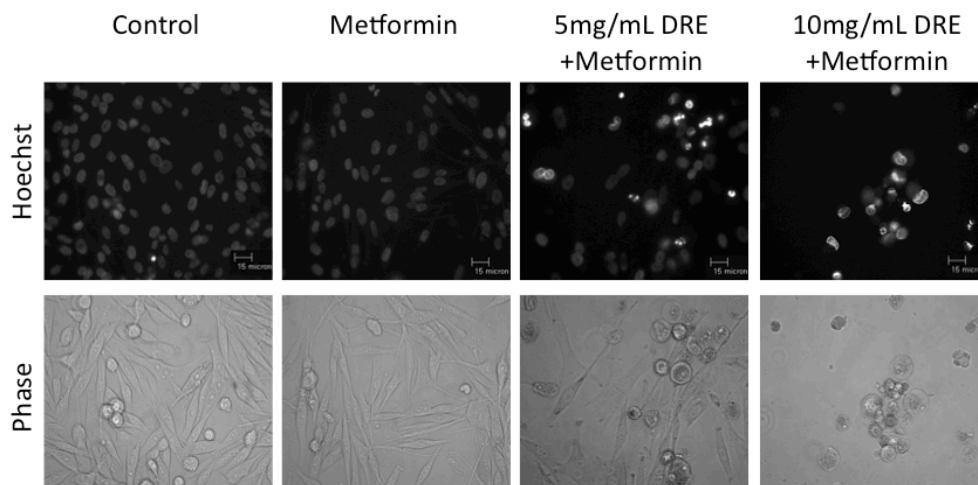


Figure 8: Combinatorial treatment of metformin and DRE on relatively - resistant G361 cells after 72 hours. A) Cell viability of the relatively resistant G361 cells. G361 human melanoma cells were seeded on 96-well plates (~3000cells/well) and treated at the indicated concentrations of DRE and 4mM metformin or 72 hours. The WST-1 dye was added to each well after every treatment period and incubated, as described in Materials and Methods. Absorbances were read at 450nm. Statistical analysis was performed using the GraphPad Prism 5.0, ** denotes a p-value <0.05; *** denotes a p-value <0.0005. **B) Induction of apoptosis in metformin-treated G361 cells by DRE.** Typical apoptotic morphology was observed in G361 cells treated with DRE starting at 5mg/mL concentrations for 72 hours. Cells were stained with Hoechst 33342 dye, before images were taken on a fluorescence microscope. Brightly stained, condensed bodies indicate apoptotic nuclei.

Discussion

Dandelion Root Extract (DRE) has thus far been used in traditional medicine as a detoxifying agent for digestive disorders, for lung, breast and uterine tumours [2], and most interestingly, to treat chronic diseases of the skin [4]. However, there has been little scientific advancement made in this field with regard to the effect of dandelion root extract on cancer, and even more so on chemoresistant, human malignant melanoma skin cancer. Previous work with *Taraxacum* have not provided much mechanistic detail with regards to apoptosis induction, instead highlighting its anti-oxidant and anti-inflammatory effects. In this study with human melanoma cells, we show that Dandelion Root Extract (DRE) is more than a worthy chemopreventative, it is fast-acting, non-toxic, and therefore specific in its targeting of human melanoma cancer cells, making it a valuable chemotherapeutic. We have investigated the induction of apoptosis in human malignant melanoma cells, and observed its long-term effects in human melanoma cancer.

The WST-1 assay (where cell viability is returned as a function of metabolic activity of mitochondrial dehydrogenases) reported reductions in A375 cell viability in a time and dose-dependent manner upon DRE treatment [Figure 1A]. By 48 hours, human melanoma A375 cells uncharacteristically showed susceptibility to apoptosis induction by DRE [Figure 1B]. These cells displayed morphological features of condensed and fragmented nuclei - typical of apoptotic cells. Based on cell viability and observation of extent of apoptosis by 48 hours, we established the

effective dose as 2.5mg/mL. We confirmed that apoptosis in A375 cells was indeed induced by DRE upon observing flipping of the phosphatidylserine from the inner to the outer leaflet of the plasma membrane at this dose [Figure 1C]. Given that DRE has traditionally been used naturopathically for a variety of ailments, we assume that it would be relative non-toxic to healthy cells. Our results show that the Normal Human Fibroblasts (NHFs) remained unaffected and healthy after a 96-hour exposure to DRE [Figure 2A and B].

Dandelion root extract has been resolved into components using chromatography techniques [6]; however, singular components themselves may not be enough to trigger a chemotherapeutic response in a chemoresistant cancer. Components may require each other to work in unison or even synergy, which is possibly why they have been effective as extracts in traditional medicines. With one of the triterpene components of DRE, Lupeol, Hata *et al.* observed a decrease in mouse melanoma differentiation [7]. This study has been supported by a two-stage skin carcinogenesis mouse model showing anti-proliferative and chemopreventative activity of this compound [19]. However, initial *in vivo* studies with *Taraxacum japonicum*, conducted by Takasaki *et al.*, concluded that it was the taraxasterol component that was the worthy cancer chemopreventative [9]. Though the extract is constituted of a myriad of compounds, we wanted to determine the resultant mechanistic effect of the combined components in specifically killing human melanoma cells.

We wanted to first determine whether DRE induced apoptosis was death receptor-mediated by measuring caspase-8 activation. Caspase-8 activation results from binding of ligands (such as Fas) to the death receptors (such as Fas receptors) on the cell surface [20]. Fas-receptor mediated apoptosis would result in recruitment and conversion of pro-caspase-8 to active caspase-8. Logically, we first chose a time point (24 hours) prior to when we had observed apoptosis (48 hours) by Hoechst staining with 2.5mg/mL DRE [Figure 1A and B]. However, this proved to be too late and it was found that upon DRE treatment, caspase-8 activation occurred within minutes, peaking around 30 minutes after treatment [Figure 3]. Ariza *et al* have previously shown that A375 cells do, express the Fas receptor, which strengthens our theory of activation of an extrinsic apoptotic pathway [21].

Judging by the diminished activity of the mitochondrial dehydrogenases of the WST-1 assay [Figure 1A], we also visualized dissipation of the mitochondrial membrane potential at 24 hours of treatment using JC-1. This indicates that the mitochondria is depolarized early on, and this in turn agrees with the induction of apoptosis that we observe at 48 hours of treatment, following the dissipation.

To ascertain whether this effect of DRE on the mitochondria is a cause or result of upstream caspases, we wanted to determine if DRE had the capability of targeting the mitochondria directly and independently of the extrinsic pathway. Though there was indeed an increase in the levels of ROS for DRE-treated mitochondria, the rate of ROS production did not differ between the effective and sub-effective doses [Figure 5]. This indicated that DRE action is not only cell death receptor-mediated, but that

its effect on the mitochondria may not be purely causative, but a consequence of direct mitochondria targeting and even possible cross talking between the extrinsic and intrinsic pathways. More importantly, an increase in ROS production indicates pro-oxidant behaviour of DRE on cancer cell mitochondria, which is contrary to the anti-oxidant convictions of traditional medicine and previous studies on Taraxacum extracts citing reductions in NO, ROS, RNS and COX-2 [10, 11] in mouse macrophages. This duality in Taraxacum's operation may depend on the cell's nature – normal vs. cancerous – further underlining Taraxacum's ability to distinguish between these cells.

Now that DRE's efficacy in specifically killing human melanoma cells has been proven, we needed to determine if cells retained the signal to commit apoptosis post-treatment in drug-free media [Figure 6A and B]. The cell count representing negative Trypan blue staining indicates membrane un-permeabilized cells- viable and also apoptotic cells [Figure 6A]. Hoechst images corroborate the Trypan counts by revealing brightly stained nuclei after 96 hours mostly at 5mg/mL DRE. These cells therefore, have retained the signals to commit suicide long after the drug has been removed, making it a worthy chemotherapeutic [Figure 6B].

Upon comparison of A375 melanoma with G361, we found that the latter did not respond to the same doses and only started to respond, though minimally, to DRE at much higher concentrations (10mg/mL) - about four times the effective concentration for A375 [Figure 7A and B]. Since the WST-1 assay showed increased susceptibility of only A375 to DRE [Figure 1A], measured at the mitochondrial level,

differences between these cell lines could highlight the mechanism by which DRE is acting to induce programmed cell death in one cell line (A375) while being resisted by the second (G361). According to Su *et al*, in a microarray study comparing different melanoma cell lines for gene expression, it was found that in G361 and A375s have varying levels of antioxidant and anti- and pro-apoptotic genes that are expressed [24]. For example at basal conditions, the anti-oxidant, pro-survival gene - ATOX1 - is up-regulated, and the pro-apoptotic gene -CASP4- is down-regulated in G361, but is un-induced in A375 in both cases. Quantification of gene expression using qRT-PCR, Su *et al*, showed significant up-regulation in 3 anti-apoptotic genes (PHB, PPP2R1B and OPA) and the antioxidant gene for glutathione reductase (GSR). We could speculate that a combination of these factors could contribute to why G361 does not respond to DRE treatment, and by eliminating these factors; we could possibly determine how DRE might therefore act in A375 human melanoma cells.

With the relatively resistant G361 cells not responding to DRE treatment, we used the anti-type-II diabetes drug, metformin, to sensitize the cells. The energy metabolism of cancer cells being different from normal cells potentiates this difference as a point of specific vulnerability [14]. Metformin acts as a metabolism interfering compound that debilitates cancer cells, and the case of G361 resistant melanoma cells, combining DRE with metformin reduces cell viability at even lower doses [Figure 8A and B]. To this date work on human melanoma cells treated with metformin has been unprecedented.

The dandelions that we had collected for this investigation were harvested in the month of May. Previous studies with Canadian dandelion have stated that during Autumn senescence, there is a accumulation of amino acids in the roots, but the levels of amino acids diminish in Spring, resulting in fluctuations between asparagine and glutamine across the seasons [25]. This is indicative that our extract is not primarily amino acid based.

As mentioned before, various components like Lupeol have been considered as chemo-preventatives for cancer. We are yet to determine the effect of each of the individual components (such as the family of triterpene alcohols and phenolic acids – found in the roots - and cinnamic acids, flavinoids and coumarins- that are found in the leaves) [26] but we believe that the compounds in DRE most likely work in synergy with each other to produce the aforementioned resultant effect. In this study, we are narrowing on the therapeutic potential of dandelion root extract rather than its caliber as a prophylactic agent. We believe that this non-toxic extract can undergo precipitous translation from bench top to bedside, with dandelion products that are already commercially available in the form of tea and supplements. Traditional therapeutics have provided us a new scope for harnessing the potential of natural extracts in modern medicine; the efficacy of DRE is only fully being realized now as a chemotherapeutic against aggressive chemoresistant cancers.

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Chapter 4

*Chemo-resistant melanoma sensitized by tamoxifen to low dose
curcumin treatment through induction of apoptosis and
autophagy*

Abstract

Melanoma is the deadliest form of skin cancer, which is notoriously aggressive and chemo-resistant, and for which there is little effective treatment available if it goes undetected. Curcumin from the tumeric spice (*Curcuma longa*) has long been used in Southeast Asian medicine to alleviate ailments and cure an array of diseases and disorders. It possesses anti-inflammatory, anti-oxidant and most importantly anti-carcinogenic activity. There have been contradictory reports discussing the efficacy of curcumin-induced death on melanoma. In this report we show that curcumin does induce apoptosis in A375 and the relatively resistant G361 malignant human melanoma cell lines at higher doses. Tamoxifen is an estrogen receptor (ER) blocker that is used for ER positive breast cancer treatment. Recently, tamoxifen has been shown to directly target the mitochondria. Given that curcumin is a pro oxidant and tamoxifen can act on mitochondria, we ask whether the combinatorial treatment could result in synergistic induction of apoptosis in chemo-resistant melanoma. Our results show a corresponding increase in phosphatidyl serine flipping, mitochondria depolarization and reactive oxygen species (ROS) generation by the combined treatment of 10 μ M tamoxifen and 10 μ M curcumin. Interestingly, there was significant induction of autophagy along with apoptosis following the combined treatment. Importantly, non-cancerous cells are unaffected by the combination of these non-toxic compounds. However, once exposed to low doses of this co-treatment, melanoma cells still retain signals to commit suicide even after removal of the drugs. This combination provides a non-toxic option for combinatorial chemotherapy with great potential for future use.

Introduction

Melanoma is the deadliest form of skin cancer and one with increasingly higher rates of incidence in young adults in North America. Currently there is no effective form of chemotherapy to combat this notoriously chemo-resistant cancer, and especially the metastasized tumours. Surgical removal of the tumour is the most successful form of treatment, curing more than 90% of cases only if the tumour is still local and is detected in its primary stages [1].

Though many chemotherapeutics are derived from natural sources (e.g. paclitaxel), there is a new wave of natural health products (NHPs) emerging that are non-toxic to normal non-cancerous cells, but are highly effective against cancer cells. One such compound that has generated a lot of interest in cancer research is curcumin. Curcumin is a compound in turmeric, which originates from the same family as ginger, and has been used in traditional medicine for thousands of years in Southeast Asia. Traditional Ayurvedic medicine has harnessed curcumin's properties in turmeric as an anti-rheumatic alleviating sinusitis, for respiratory problems, hepatic disorders and even anorexia; in the Far East it has been used as an anti-inflammatory². Scientific evidence accumulated over the years confirms that curcumin possesses anti-oxidant, anti-microbial, hepatoprotective, and most importantly, anti-carcinogenic activity [2, 3, 4].

Its chemotherapeutic capability has already been proven using various tumorigenic cell lines. Syng-ai *et al.*, have shown that curcumin effectively induced apoptosis in the HepG2 hepatocellular carcinoma and two breast carcinoma cell

lines (MCF-7 and MDA-MB) [5]. Mitochondria are a central component of apoptosis. Direct disruption of mitochondria, causing dissipation of the membrane potential and even reactive oxygen species generation, will result in release of pro-apoptotic proteins, thereby committing cells to death. Curcumin-induced apoptosis was also found to be associated with ROS generation in colon cancer (HT29 and HCT116) cells, hepatomas (Hep 3B and HepG2), submandibular carcinoma and a transformed human skin cancer cell line (COLO16) [6-9]. Curcumin has been shown to generate ROS that resulted in mitochondrial DNA (mtDNA) damage in HepG2 cells [10]. Melanoma, along with cervical, prostate, lung and kidney cancer cell lines, exhibit resistance to the cytotoxic effects of curcumin treatment [11]. Contradictory to reports of curcumin-resistance, certain human melanoma cell lines (including C32, G-361, and WM 266-4) were found to be susceptible to curcumin-induced, death-receptor mediated apoptosis, independently of p53 and with suppression of NF- κ B [12, 13]. It has been speculated that this multi-pathway, non-DNA targeting, non-toxic drug is able to facilitate apoptosis induction in a mitochondria-centralized manner. Similarly, there are other compounds/drugs like curcumin that are also non-genotoxic, as well as non-toxic to normal cells, such as tamoxifen.

The breast cancer drug, tamoxifen, is used as a non-genotoxic, safe mode of adjuvant therapy to treat and prevent breast cancers, and has been used for the past 40 years. Until recently, tamoxifen was thought to target only the estrogen receptor of breast cancer cells. In addition to this receptor, mitochondria have also been shown to be a direct target of tamoxifen [14]. In previous studies, both Siedlakowski and co-workers and our own work have shown that tamoxifen directly targets the

mitochondria of estrogen receptor positive and negative breast carcinoma, and importantly, the mitochondria of A375 human melanoma cells, sensitizing all three cell lines to pancratistatin-induced (an *Amaryllidaceae* alkaloid from the *Hymenocallis littoralis*) apoptosis [15, 16]. Until now, the combinatorial effect of curcumin and tamoxifen, two known mitochondria-targeting compounds, has not been studied.

Recent studies indicate that autophagy might be playing a role in resistance to current cancer therapy [17]. Autophagy (programmed cell death II-PCD II) occurs naturally in cells during periods of starvation or cellular stress and can be either pro-survival or pro-death. The stress of cytotoxic insults from therapy may cause cells to adapt to the microenvironment, which is a result of pro-survival autophagy. When there is extreme stress, a massive autophagic response ensues, resulting in autophagic death (pro-death). Tamoxifen is a known inducer of autophagy [17].

In this chapter, therefore, we show for the first time that curcumin in combination with tamoxifen, both at low dose concentrations, induces a synergistic increase in cell death than either drug alone. Moreover, this synergistic combination was non-toxic to healthy, normal cells, while remaining fatal to otherwise chemo-resistant human melanoma cells long after the drugs were removed. Furthermore, cell death as a result of curcumin and tamoxifen co-treatment may not be primarily due to apoptosis but possibly autophagy.

Materials and Methods

Cells and Reagents

Human malignant melanoma cells lines A375 and G361 were obtained from ATCC (Manassas, VA, USA). A375 human melanoma cells were grown and cultured at 37 °C and 5% CO₂ in RPMI-1640 media containing L-glutamine and NaHCO₃ (Sigma-Aldrich, Oakville, ON, Canada) completed with 10% Fetal Bovine Serum (FBS) (Sigma, Canada) and 10 µg/mL gentamycin (Gibo, Canada). G361 human melanoma cells were cultured in McCoy's Medium 5a modified with L-glutamine (Gibco, Canada) and also completed in the same manner as the RPMI 1640 media.

Normal human Fibroblasts (NHFs) were purchased from the Coriell Institute for Medical Research, USA. NHFs were cultured in Earle's Minimum Essential Medium (Sigma-Aldrich, Oakville, ON, Canada) completed with 15% FBS, 2 mM L-glutamine, 10 µg/mL gentamycin, vitamins, and essential and non-essential amino acids (Gibco, Canada).

WST-1 Assay

Cell viability was assessed using the WST-1 reagent (Roche Applied Science, IN, USA). Cells were plated at a fixed number (A375 plated at ~1000cells; G361 plated at ~3000 cells) in a 96-well tissue culture plate. After 24 hours the cells were treated with curcumin and/or tamoxifen at the indicated concentrations. WST-1 reagent was added to the wells after the treatment period, and incubated at 37°C for 4 hours. The plates were then read at 450nm on a Wallac Victor³™ 1420 Multilabel Counter (Perkin

Elmer[™], Ontario, Canada). Absorbance readings are expressed in terms of cell viability as percent control (untreated cells).

Cellular Staining

Cells treated with curcumin and/or tamoxifen for the specified time periods were stained with Hoechst 33342 dye (final concentration 10 μ M) as previously published [16], to image the nuclear morphological features. Apoptotic cells are characterized by condensed, brightly stained nuclei. Non-apoptotic cells are not condensed and do not stain brightly. Cells were imaged with the dye using a fluorescent microscope (Leica DM IRB); pictures were taken at 40X objective.

Cells stained for autophagy using monodansylcadaverine (MDC) (which can stain the autophagic vacuoles through interactions with lipids and ion-trapping) [18] were grown on coverslips. Cells were treated as indicated and post-treatment, cells were incubated in 0.1mM MDC as per a previously published protocol [19].

Annexin-V Binding Assay

To confirm apoptosis induction, the Annexin-V binding assay was performed after the indicated treatment periods using a purchased kit and the manufacturer's protocol (Sigma-Aldrich, Oakville, ON, Canada). Post-treatment cells were washed twice with 1X PBS and re-suspended in Annexin-V binding buffer (10mM HEPES, 10mM NaOH, 140mM NaCl, 1mM CaCl₂, pH 7.6). Annexin-V-FITC conjugate (1:50) was added to the cells and incubated for 15 minutes at room temperature. Cells

were observed and imaged under a fluorescent microscope (Leica DM IRB); images were taken at 40X objective.

Caspase-8, -9 and -3 Activity

Caspase-8, -3 and -9 activity was assayed using a previously published protocol [16] and manufacturer's procedure (Enzyme System Products, USA). Cell lysates were prepared using A375 human melanoma cells and incubated for 1 hour separately with caspase-3 fluorogenic substrate (DEVD-AFC) in DEVD buffer, caspase-8 fluorogenic substrate (IETD-AFC) in IETD or caspase-9 fluorogenic substrate (LEHD-AFC) in the same buffer at 37°C in 96-well opaque microtitre plates. All substrates were purchased from Calbiochem. AFC fluorescence was measured at 400 nm excitation and 505 nm emission using spectrofluorometer (SpectraMax Gemini XS). Caspase activity was measured expressed in terms of relative fluorescence units per μg protein, and protein concentration was determined using Bradford Assay (BioRad); Bovine Serum Albumin (BSA) was used as a standard.

Observation of Mitochondrial Membrane Potential Depolarization

Human melanoma A375 cells were seeded on sterile coverslips in 6-well tissue culture plates, and treated at the indicated concentrations of curcumin and/or tamoxifen. After curcumin and/or tamoxifen treatments, the cells were incubated for 1 hour with JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-

benzamidazolocarboxyanin iodide) at a final concentration of 0.5 μ M at 37°C. Cells were observed and imaged using a fluorescent microscope (Leica DM IRB); images were taken at 40X objective.

Measurement of Mitochondrial Reactive Oxygen Species

Mitochondria were isolated from A375 cells based on a previously published protocol [20]. The mitochondria were treated directly with DRE in 96-well opaque microtitre plates. Amplex Red (5 μ M) and horseradish peroxidase (HRP) were added to the mitochondria immediately after direct treatment. The fluorescent Amplex Red product – resorufin - was measured at 530nm excitation and 580nm emission using a spectrofluorometer (SpectraMax Gemini XS). Readings were expressed as relative fluorescence units (RFU).

Post-Treatment Cell Revival

A375 cells pre-treated curcumin and/or tamoxifen for 96 hours, were trypsinized, and re-plated in drug free RPMI1640 media, and incubated for 96 hours at 37 °C and 5% CO₂. Cells were stained using Hoechst 33342 dye and imaged using a fluorescent microscope (Leica DM IRB); pictures were taken at 40X objective. Cells were also collected and counted using Trypan blue exclusion dye.

Results

Effect of curcumin on human melanoma cells

Previous scientific reports have proven curcumin to be effective as an anti-cancer compound in many different cancers including neurological, breast, ovarian, lung, and gastrointestinal cancers [21, 22]. To determine the effect of curcumin on A375 human malignant melanoma cells, cells were treated for 72 hours at various concentrations ranging from 1 μM to 100 μM , and cell viability was measured using the WST-1 assay. This assay measures cell viability as a function of cellular metabolic activity. A375 cell viability reduced at concentrations at, and exceeding, 10 μM [Figure 1A, page 124]. To determine the effect of 10 μM curcumin on the growth kinetics of A375 human melanoma cells, cells were treated for 24, 48, 72 and 96 hours, and counted using the Trypan blue exclusion assay. Compared to control there is growth inhibition due to curcumin treatment over time [Figure 1B, page 124].

Figure 1

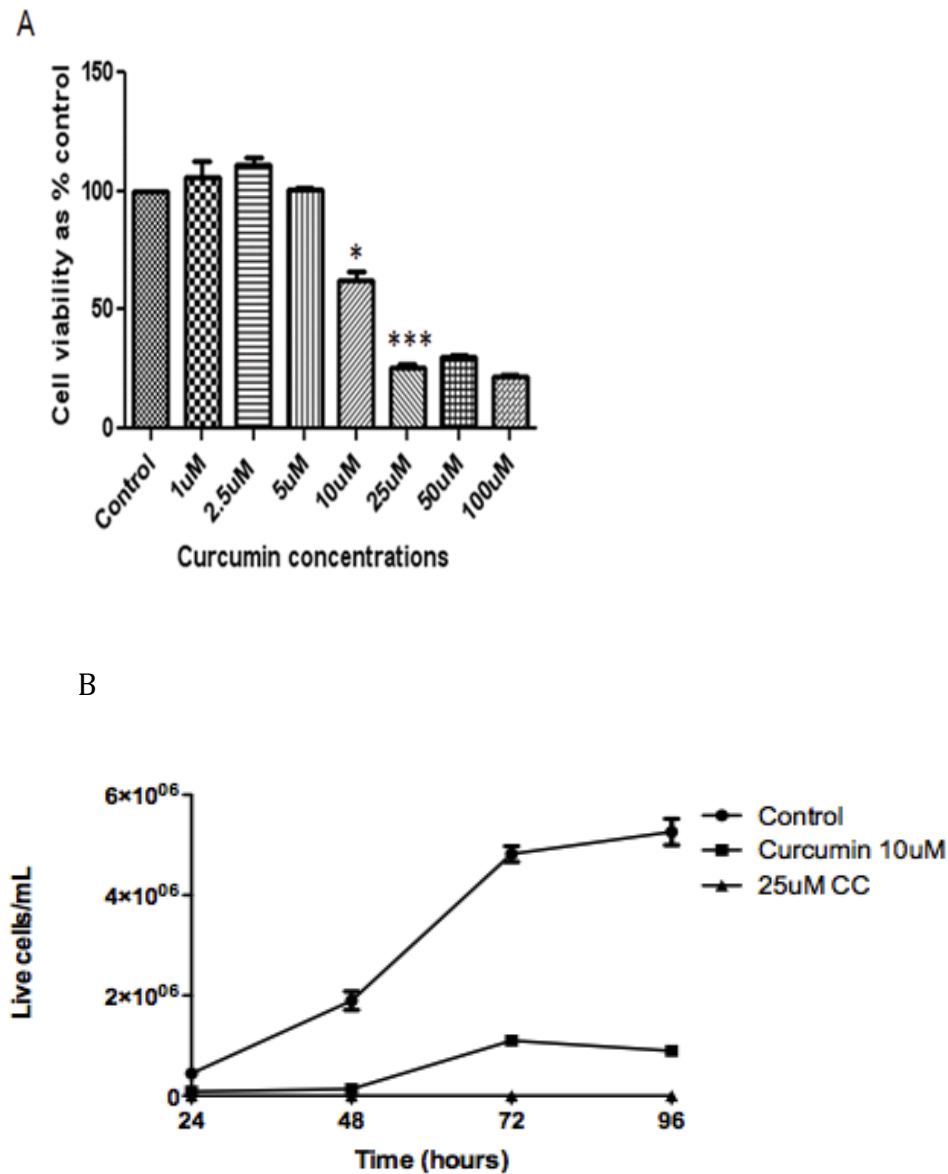


Figure 1: Effect of curcumin treatment on human melanoma A375 cells. A) Cell viability after 72 hours curcumin treatment. A375 cells were seeded in a 96-well plate and treated with varying concentrations of curcumin for 72 hours. The WST-1 dye was added after treatment, as described in Materials and Methods, and absorbances were read at 450nm. **B) A375 cells treated over time with 10 and 25 μ M curcumin.** Cells were treated with 10 μ M curcumin for 24, 48, 72 and 96 hours, and were counted using Trypan blue exclusion dye. Statistics performed using GraphPad Prism version 5.0; * denotes a p-values < 0.05 compared, and *** denotes a p-value < 0.0001 to control.

To ascertain whether these cells were dying by apoptosis, A375 cells treated with 10 μ M and 25 μ M curcumin for 72 hours and were stained with Hoechst 33342 dye. This dye stains the DNA and distinguishes brightly stained, condensed apoptotic nuclei from dimly stained healthy nuclei. With an increase in curcumin concentration we observed an increase in the number of apoptotic nuclei, and this mode of cell death was confirmed with the Annexin-V binding assay. This stain indicates the flipping of the phosphatidyl serine to the outer plasma membrane (a positive biochemical marker of apoptosis). [Figure 2, page 126] exhibits an increase in cells positive for the phosphatidyl serine-flipping i.e. they are more apoptotic at 25 μ M than they are at 10 μ M curcumin. The phase contrast images corroborate the apoptotic events, as seen by the condensed cellular morphology of the apoptotic cells.

Figure 2

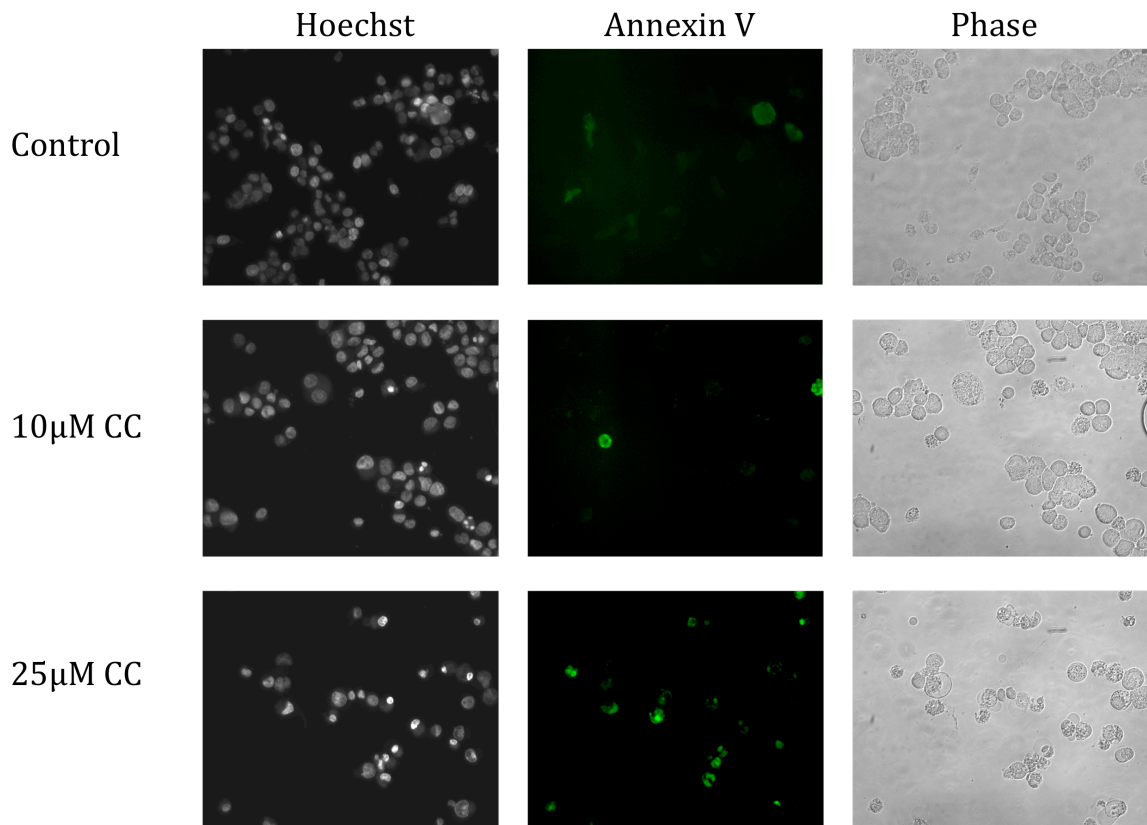


Figure 2: Apoptosis induction by curcumin in A375 cells. A375 cells were treated with curcumin at the indicated concentrations for 48 hours. Nuclei were stained with Hoechst 33342 dye to determine if cell death was by apoptosis or not. The Annexin-V binding assay was also performed to confirm apoptosis (as described in Materials and Methods) and cells were imaged on a fluorescent microscope. Images were taken at 40X objective.

Given that curcumin is known to possess anti-oxidant, free-radical scavenging properties [23], we aimed to determine its effect on ROS production from isolated mitochondria of A375 cells. Measurement of H₂O₂ production by Amplex Red reagent (as described in Materials and Methods) showed an increase in ROS production from 10 μ M to 25 μ M curcumin against control [Figure 3].

Figure 3

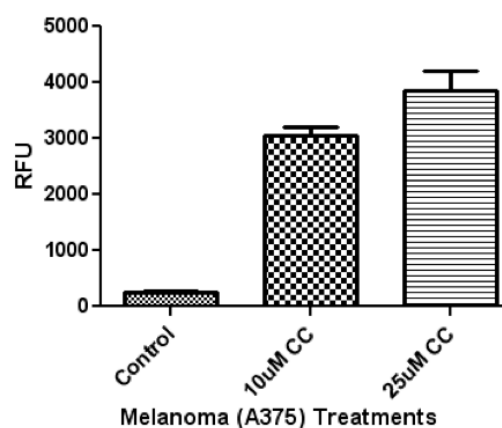


Figure 3: Reactive Oxygen Species (ROS) generation from isolated A375 mitochondria after curcumin treatment. Mitochondria isolated from A375 cells were treated directly with curcumin at the indicated concentrations and ROS was measured using Amplex Red substrate in presence of horseradish peroxidase (HRP) as described in Materials and Methods. Statistics performed using GraphPad Prism version 5.0; * denotes a p-values < 0.05 compared to control.

This increased production of ROS indicates destabilization of the mitochondria, which suggests that curcumin induces mitochondria-mediated apoptosis to kill cancer cells. Destabilization of the mitochondria leads to release of pro-apoptotic factors like cytochrome *c*, which in turn activates caspase-9. This is shown to occur at relatively higher doses of curcumin treatment (25 μ M) within 48 hours [Figure 4].

Figure 4

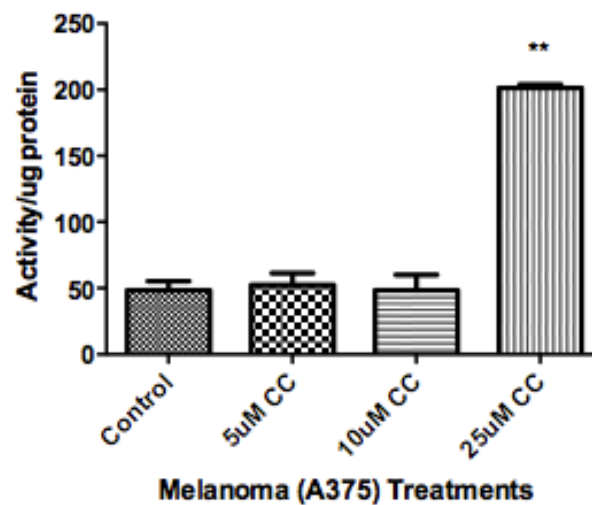
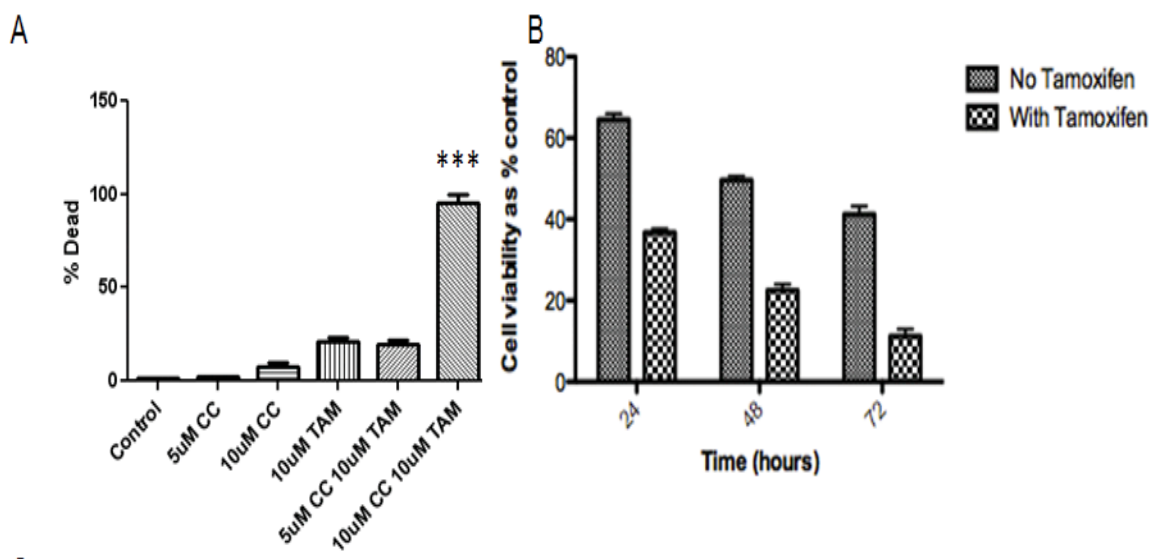


Figure 4: Activation of caspase-9 in A375 cells by curcumin after 48 hours. Cells were treated at the indicated doses for 48 hours after which cells were lysed and incubated with caspase-9 substrate, as described in Materials and Methods. Statistical analysis was performed using GraphPad Prism 5.0 and ** denotes p-values <0.01.

Effect of Tamoxifen on curcumin-treated melanoma

Recent studies have indicated that the breast cancer drug tamoxifen has the ability to not only target the estrogen receptor but can directly target the mitochondria [14]. When tamoxifen was combined with 10 μ M curcumin, the viability of A375 cells dropped dramatically by 72 hours as indicated by the Trypan blue exclusion assay [Figure 5A] (figure legend provided on page 130); this reduction is also observed over time using the WST-1 assay [Figure 5B]. Decreased viability of A375 cells was attributed to induction of apoptosis, as cells treated with curcumin and tamoxifen for 48 hours had classical apoptotic nuclear morphology and were Annexin-V positive [Figure 5C, page 130].

Figure 5



C

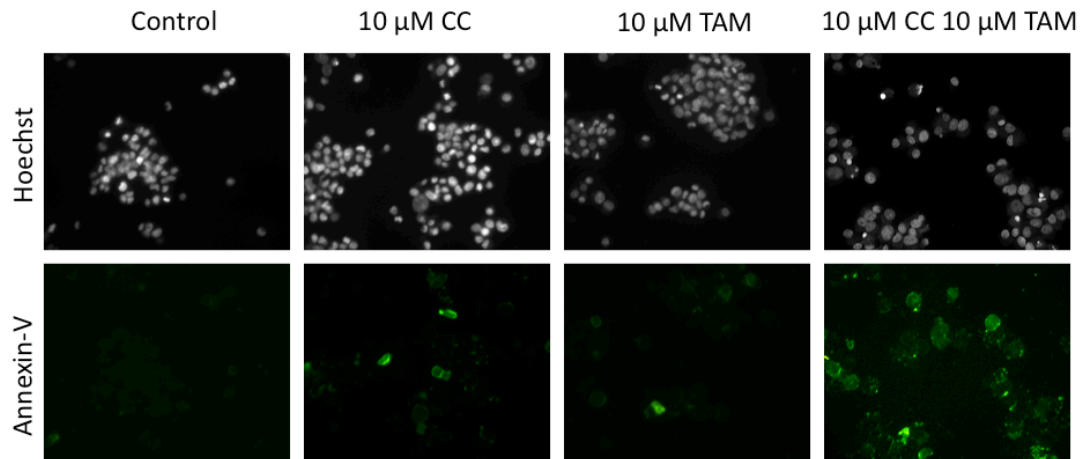
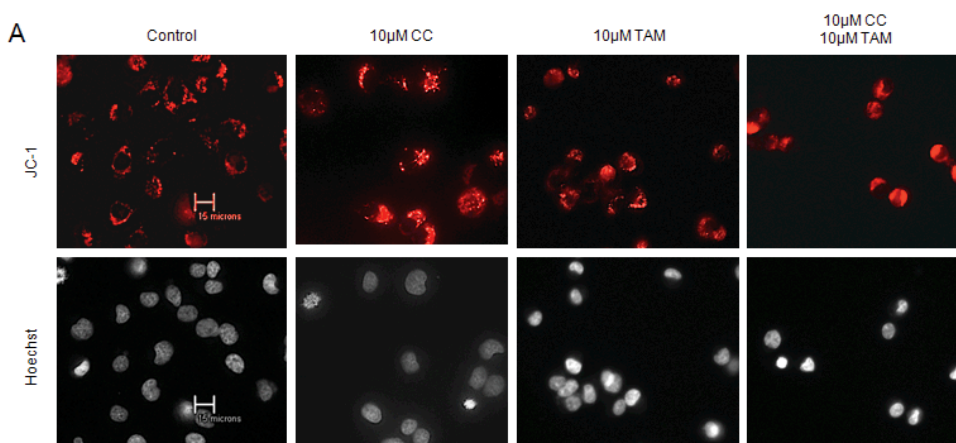


Figure 5: Co-treatment of A375 human melanoma cells with curcumin and tamoxifen. A) Quantification of dead A375 cells after curcumin-tamoxifen treatment by Trypan staining. A375 melanoma cells were treated at the indicated concentrations of curcumin and tamoxifen for 72 hours. Cells were collected and counted using the Trypan exclusion assay. **B) Effect of curcumin and tamoxifen on A375 viability by WST-1.** A375 cells were plated for WST-1 assay and treated for 24, 48 and 72 hours with 10 μ M CC in the presence/absence of 10 μ M TAM. **C) Apoptosis induction by curcumin and tamoxifen in A375 cells after 48 hours.** Cells treated at the indicated concentrations of curcumin and tamoxifen for 48 hours, were stained with Hoechst 33342 dye to observe apoptotic nuclei and then with Annexin-V Alexafluor 488 to observe flipping of the phosphatidyl serine. Cells were imaged on a fluorescent microscope. Images were taken at 40X objective; the scale bar represents 30 microns. Statistics performed using GraphPad Prism version 5.0; *** denotes a p-values < 0.001 compared to control.

To examine the effect of this combination on the mitochondria, the JC-1 stain was applied to curcumin and/or tamoxifen treated cells. The JC-1 dye aggregates in healthy mitochondria forming punctate marks such as those seen in the control [Figure 6A] (figure legend on page 132). These punctate marks appear faded upon treatment with singular treatments of 10 μ M curcumin and 10 μ M tamoxifen compared to control, and by 24 hours the co-treatment shows even greater dissipation of the potential. Based on the previous result, the mitochondria become vulnerable to curcumin-tamoxifen co-treatment, it was yet to be determined whether the combination could directly destabilize the mitochondria. Isolated mitochondria from A375 cells were treated with curcumin and tamoxifen to measure ROS production. Evidently, co-treatment did indeed cause direct destabilization of the mitochondria and in fact, co-treatment generated significantly more ROS than either treatment alone [Figure 6B]. Figure 6B and the legend for Figure 6 is given on page 132.

Figure 6



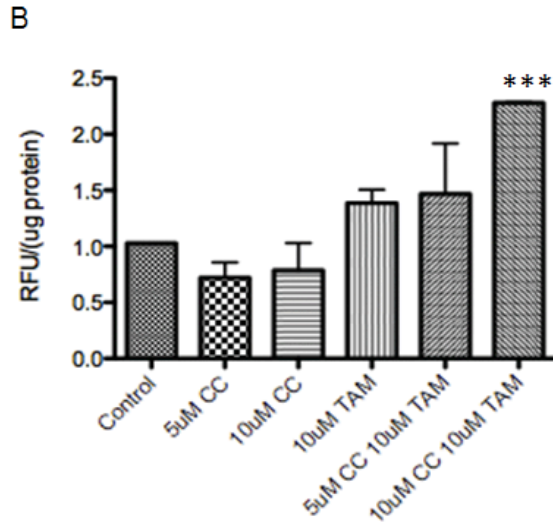


Figure 6: Effect of curcumin and tamoxifen co-treatment on the mitochondria.
A) Changes in A375 mitochondrial membrane potential observed by JC-1 staining after 24 hours treatment. A375 cells were seeded on cover slips and treated with curcumin and tamoxifen at the indicated concentrations for 24 hours. Cells were then stained with JC-1 dye (as described in Materials and Methods) and imaged on a fluorescent microscope to observe changes in the mitochondrial membrane potential. Images were taken at 40X objective; the scale bar represents 30 microns. **B) A375 mitochondrial ROS generation after curcumin and tamoxifen treatment.** Mitochondria isolated from A375 cells were treated directly with curcumin and tamoxifen at the indicated concentrations to measure ROS generation. ROS was measured using Amplex Red substrate in presence of horseradish peroxidase (HRP) as described in Materials and Methods. Statistics performed using GraphPad Prism version 5.0; ***denotes a p-values < 0.0001 compared to control.

To further investigate the mechanism by which the co-treatment is acting, caspase activation was studied in A375 melanoma cells. It was found that at the end of 48 hours treatment, neither of the caspases- 8, 9 or 3, were activated compared to the control [Figure 7A, B and C]. Pre-treatment of cells with the pan-caspase inhibitor, Z-VAD-FMK, followed by treatment with curcumin and/or tamoxifen did not significantly affect the levels of apoptosis, which correlates with the caspase activity assays, suggesting that co-treatment induced cell death is caspase-independent (not shown).

Figure 7

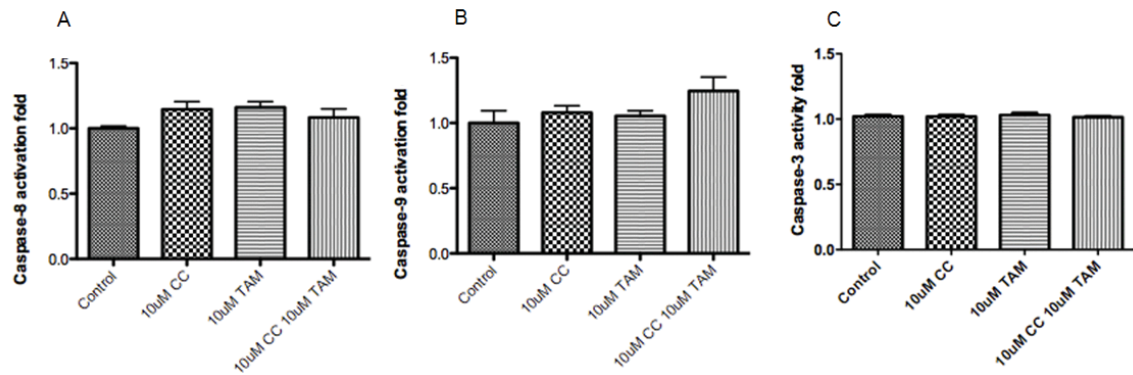
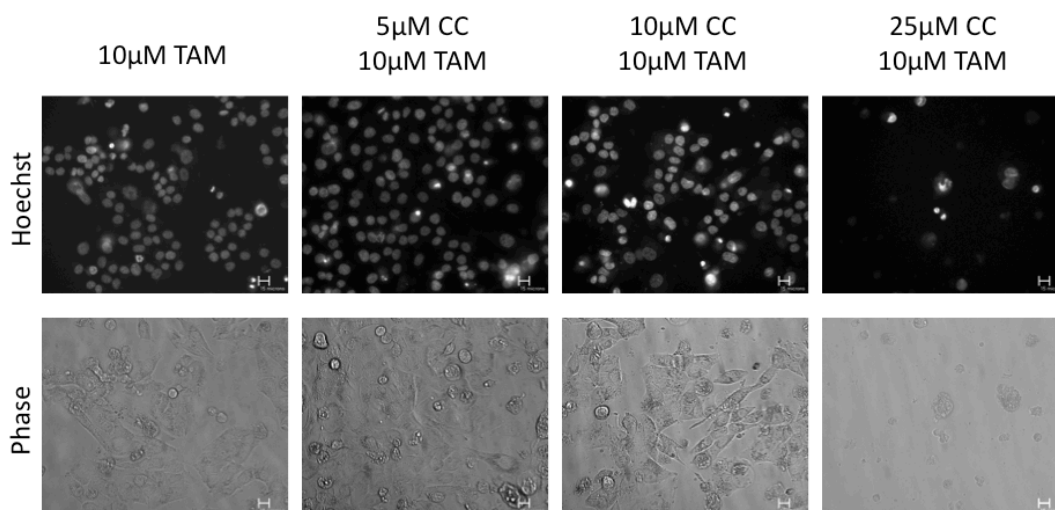


Figure 7: Caspase-8, -9 and -3 activity measurement after 48 hours curcumin and tamoxifen co-treatment. A) Caspase-8, B) caspase-9 and C) caspase-3 AFC-conjugated substrates were incubated with lysate of curcumin and/or tamoxifen pre-treated (48 hours) A375 cells and fluorescence from the cleaved AFC fluorophore, from the respective substrate, was measured on the Spectramax Gemini as described in Materials and Methods.

The co-treatment also induced apoptosis in the relatively more resistant G361 cell line, albeit at 25 μ M curcumin rather than at 10 μ M [Figure 8A] (figure legend provided on page 135). Regardless, the combination of 10 μ M curcumin with 10 μ M tamoxifen still resulted in a greater percentage of dead cells than either treatment alone [Figure 8B, page 135].

Figure 8

A



B

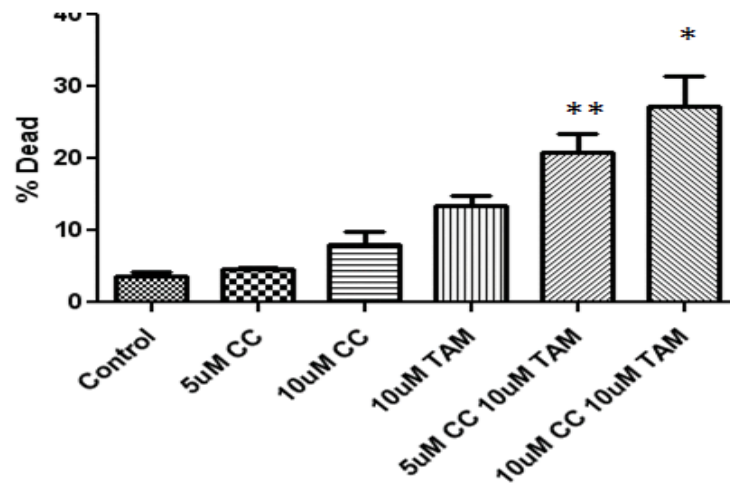
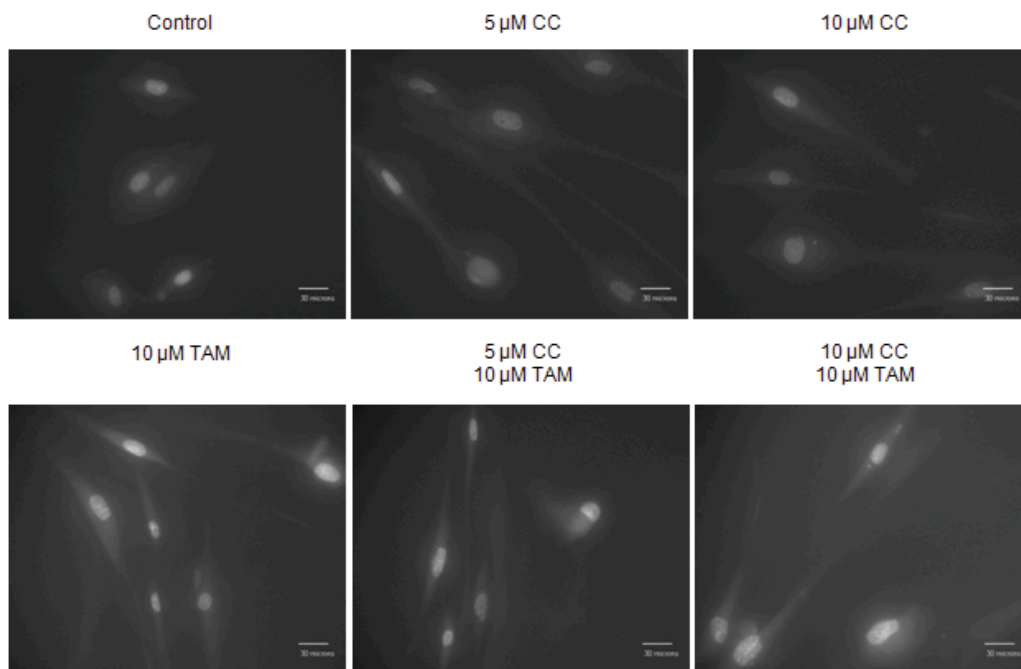


Figure 8: Observation and quantification of G361 human melanoma cells after 72 hours curcumin and tamoxifen treatment. A) Observation of G361 cells by Hoechst. G361 human melanoma cells were treated at the indicated concentrations of curcumin and tamoxifen for 72 hours. Cells were stained with Hoechst 33342 dye and imaged on a fluorescent microscope. Images were taken at 40X objective; the scale bar represents 30 microns. **B) Quantification by Trypan exclusion assay.** The G361 cells were collected and counted, after 72 hours treatment, using the Trypan exclusion assay. Statistics performed using GraphPad Prism version 5.0; * denotes a p-value < 0.05 and ** denotes a p-value < 0.01, compared to control.

Normal Human Fibroblasts (NHFs) were exposed for 96 hours to single treatments and co-treatments, and showed no effect after 96 hours of treatment [Figure 9A] (figure legend provided on page 137). After 24 hours of treatment, NHFs did not exhibit a noticeable difference in mitochondrial membrane potential of treated cells compared to control cells [Figure 9B, on page 137].

Figure 9

A



B

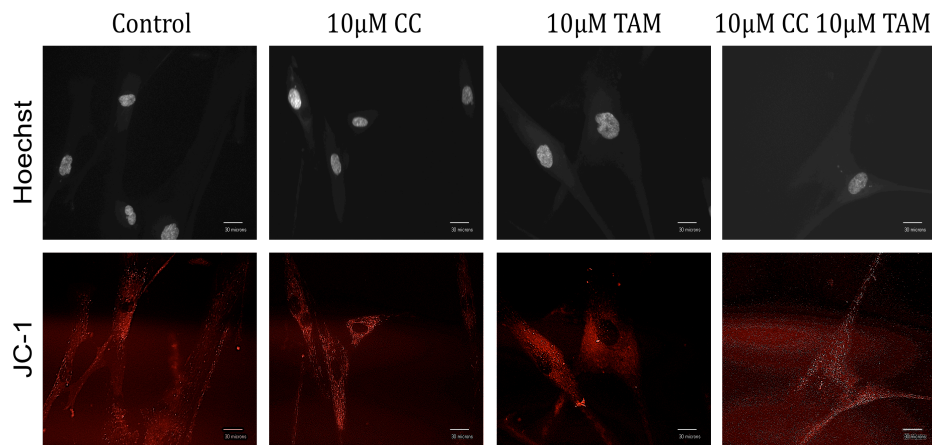
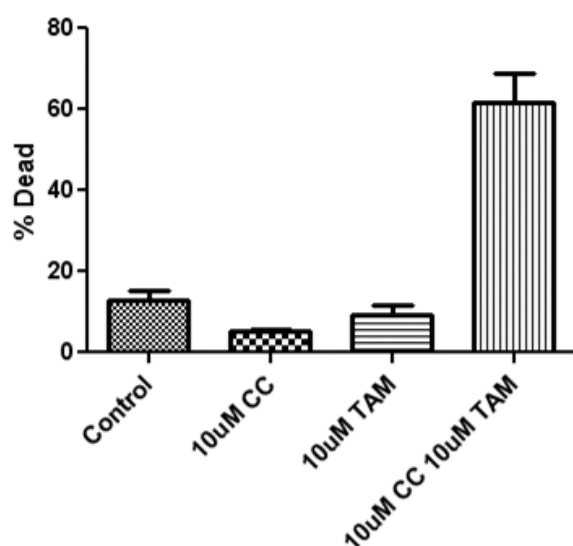


Figure 9: Effect of curcumin and tamoxifen on Normal Human Fibroblasts (NHF). A) Hoechst images of NHFs treated with curcumin and tamoxifen after 96 hours. NHFs were plated and treated at the indicated concentrations with curcumin and tamoxifen for 96 hours. Cells were then stained with Hoechst 33342 dye and imaged on a fluorescent microscope. Images were taken at 40X objective; the scale bar represents 30 microns. B) Changes in NHF mitochondrial membrane potential observed by JC-1 staining after 24 hours treatment. NHFs were seeded on coverslips and treated at the indicated concentrations for 24 hours. Cells were stained with JC-1 dye as described in Materials and Methods and images were taken at 40X objective on a fluorescent microscope; the scale bar represents 30 microns.

Considering that the combination of these two drugs has exhibited such a detrimental effect to chemo-resistant human melanoma, an evaluation in the long-term efficacy of this potential co-treatment was performed. A revival experiment was conducted whereby A375 melanoma cells were treated with 10 μ M curcumin and 10 μ M tamoxifen for 96 hours. The cells were then collected, and re-plated in drug-free media for another 96 hours before being harvested for Trypan blue counting. The co-treatment prevented growth of residual non-apoptotic cells after the initial 96 hours of treatment [Figure 10A] (figure legend provided on page 139). Hoechst staining reveals that these cells are apoptotic after 96 hours of revival [Figure 10B, page 139].

Figure 10

A



B

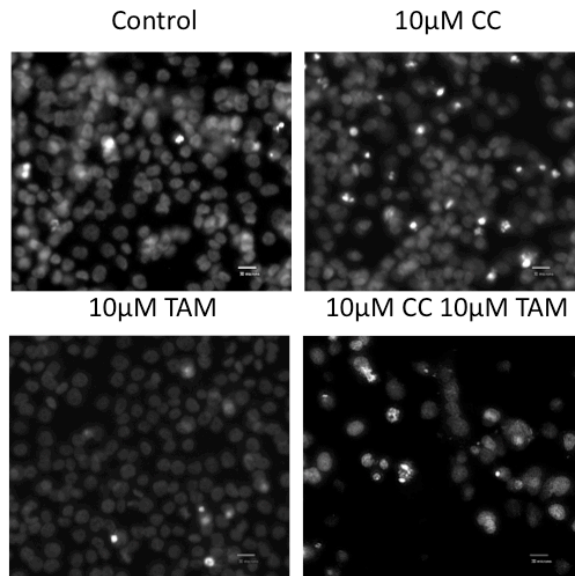
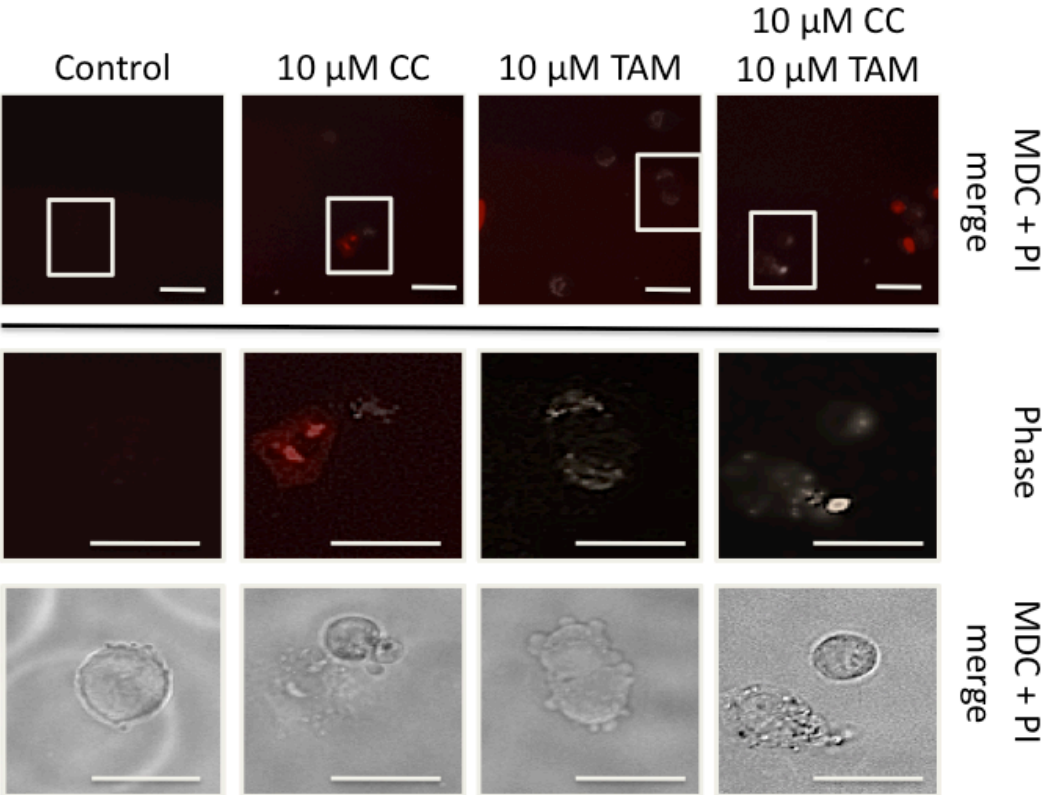


Figure 10: Observation and quantification of the effect of long-term curcumin and tamoxifen co-treatment on human melanoma cell revival. A) Quantification using Trypan Blue. A375 cells were treated for 96 hours with curcumin and tamoxifen. Cells were then collected, washed and re-plated in drug-free media and incubated for another 96 hours before cells were counted with Trypan blue. **B) Observation using Hoechst stain.** Cells were stained with Hoechst 33342 dye and imaged on a fluorescence microscope. Images were taken at 40X objective; the scale bar represents 30 microns. Statistics performed using GraphPad Prism version 5.0; *** denotes a p-values < 0.001 compared to control.

Based on the above observations whereby apoptosis was observed without caspase (-3, -8 or -9) activation, a second form of cell death was studied. Autophagic cell death occurs during cell starvation and has pathways that overlap with apoptosis pathways. Monodansylcadaverine (MDC) was used to stain cells that were treated with curcumin and/or tamoxifen. As shown in Figure 11A (page 141), brightly stained autophagic vacuoles were observed following treatment with tamoxifen, which is a known inducer of autophagy [17]. Interestingly, curcumin has also been shown to induce autophagy within 48 hours in glioma cells at concentrations of 40 μ M, by inhibiting the Akt/mTor/p70S6K pathway and activating the ERK1/2 pathway [24]. Our findings indicate that 10 μ M tamoxifen is enough to instigate an autophagic response as observed by vacuole formation by MDC staining. This finding is supported by Bursch *et al.*, who found that 10 μ M tamoxifen treatment resulted in vacuole formation within 24 hours of treatment in MCF-7 cells [25]. Here, we found that curcumin induces autophagy, although minimally compared to tamoxifen alone [Figure 11A, page 141] (figure legend is on page 142). However, the combination showed greater staining intensity i.e. greater autophagic vacuole formation, and corroborated with the phase image, which clearly shows the tiny vacuoles in the indicated cell. By 48 hours we see that the autophagic cells are dying, as the MDC clearly cross-stains the dying A375 melanoma with the propidium iodide [Figure 11B, 142]. Propidium iodide is a DNA intercalating dye that is membrane impermeable, and so cannot stain viable cells.

Figure 11

A



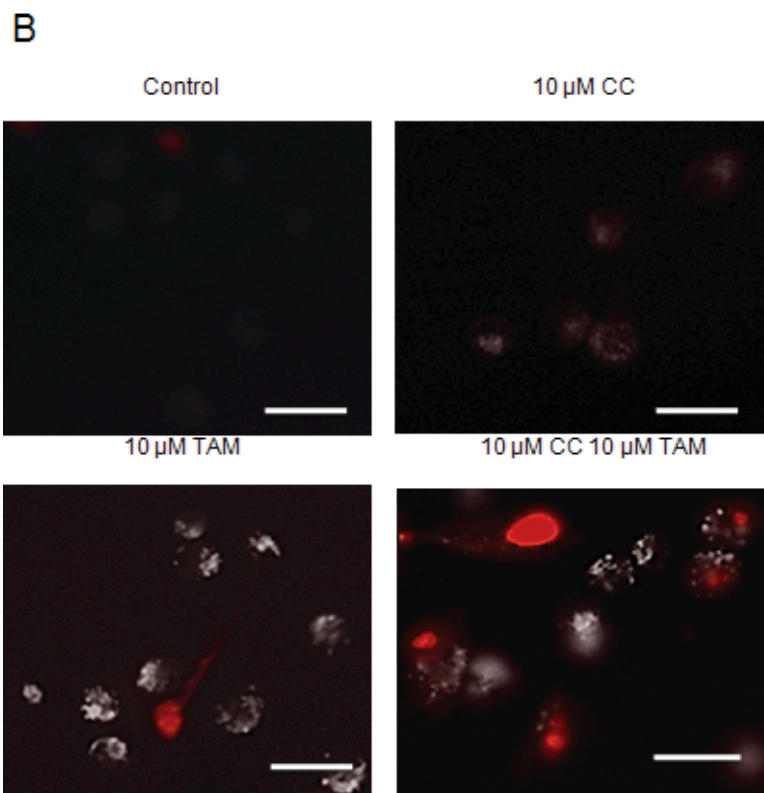


Figure 11: Determining autophagy induction by curcumin and tamoxifen treatment after 24 and 48 hours in A375 cells. A) Autophagy after 24 hours. A375 cells were grown on coverslips and treated for 24 hours at the indicated concentrations. Cells were then stained with MDC and propidium iodide as described in Materials and Methods, to observe the presence of autophagic vacuoles (brightly stained) and dead cells (red), and imaged on a fluorescence microscope. Images were taken at 40X objective; the scale bar represents 30 microns. The top panel shows the actual field image. **B) Autophagy after 48 hours.** Staining of autophagic vacuoles was repeated after 48 hours treatment.

Therefore, we can conclude that this autophagic balance has tipped from pro-survival autophagy with tamoxifen treatment alone (which is conferred by many chemotherapies including tamoxifen), to pro-death autophagy, in combination with curcumin. We have shown that there is no change in the levels of caspase activation (-3, -8 and -9) compared to control [Figure 7A, B and C], and in a separate experiment observed little change in apoptosis induction between treatments with and without the pan-caspase inhibitor (10 μ M) (data not shown). This agrees with the caspase independent trait that is associated with autophagy. In a preliminary study, a similar effect was observed in the relatively more resistant G361 cell line after 48 hours of treatment [Figure 12, page 144].

Figure 12

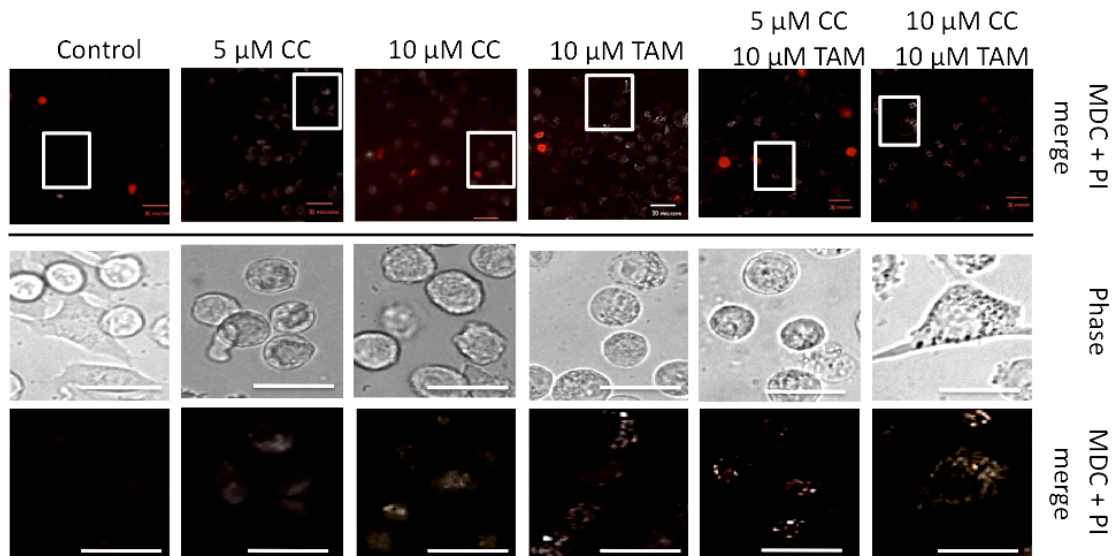


Figure 12: Determining autophagy induction by curcumin and tamoxifen in G361 cells. G361 cells were grown on coverslips and treated for 48 hours at the indicated concentrations. Cells were then stained with MDC and propidium iodide to observe autophagic vacuoles, and imaged on a fluorescence microscope. Images were taken at 40X objective; the scale bar represents 30 microns. The top panel shows the full field image. The white box represents the field that we zoom-in on in the bottom two panels.

Furthermore, in a second revival experiment (also preliminary), A375 cells were still found to retain signals to induce autophagy after 96 hours treatment and 120 hours incubation in drug-free media. As shown in figure 13, in the very last panel, autophagy was also accompanied by apoptotic cell death [Figure 13].

Figure 13

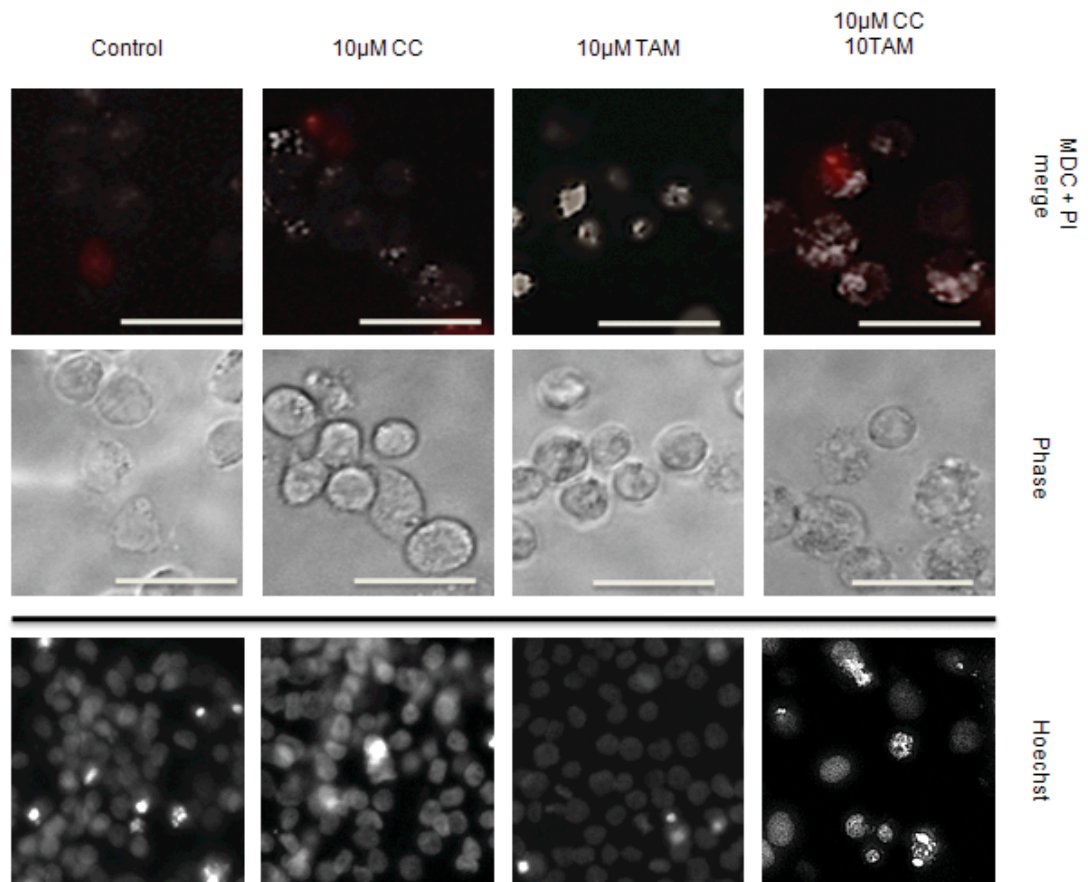


Figure 13: Autophagy induction after 96 hours curcumin and tamoxifen treatment, followed by 120-hour revival. A375 cells were grown on a cover slip and treated for 96 hours with indicated concentrations of curcumin and tamoxifen. Media-laden drugs were removed and cells were washed with PBS, before fresh, drug-media was added back into the wells. Cells were stained with MDC and propidium iodide as described in Materials and Methods, and imaged on a fluorescent microscope. Images were taken at 40X objective; the scale bar represents 30 microns. For comparison, the last panel shows the apoptotic nuclei of A375 melanoma after 96 hours treatment and 96 hours revival.

In contrast, NHFs treated for 96 hours with curcumin and tamoxifen did not show prominent autophagic vacuoles [Figure 14], though some were present after 24 hours treatment (data not shown). This indicates that the initial insult was not large enough to trigger a pro-death response. Instead, it results in a pro-survival response and this correlates with the fact that by 96 hours NHFs do not undergo apoptosis post combinatorial treatment [Figure 9A].

Figure 14

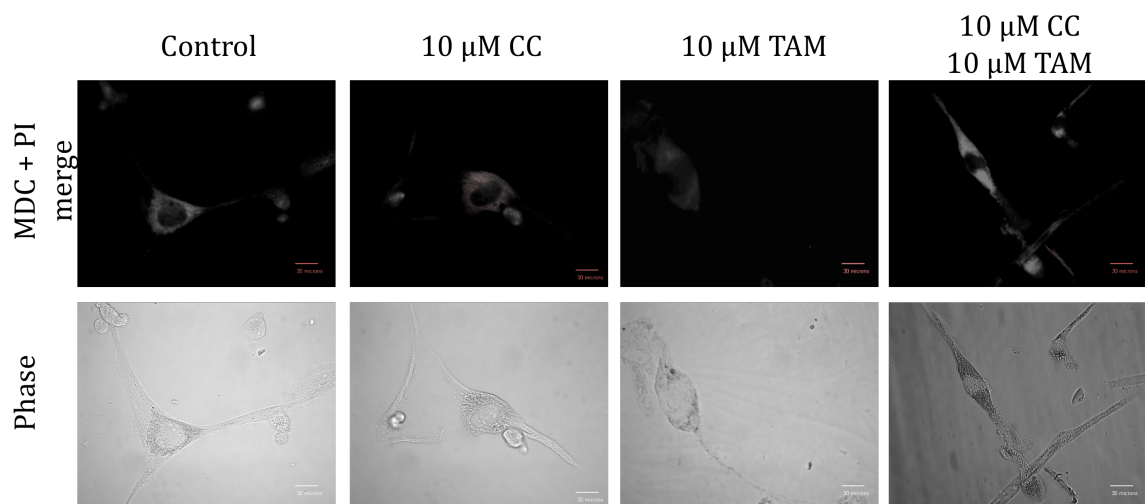


Figure 14: Lack of autophagic vacuole formation in NHFs after 96 hours treatment with curcumin and tamoxifen. NHFs were seeded on coverslips and treated at the indicated concentrations for 96 hours, following which cells were stained with PI and MDC as described in Materials and Methods. Cells were imaged on a fluorescence microscope at a 40X objective; the scale bar represents 30 microns.

Discussion

Currently, progress is being made in the field of natural health products as new compounds/extracts are coming to light as potential forms of non-toxic cancer chemotherapy. Curcumin, also known as diferuloylmethane, has garnered much attention as a non-toxic anti-cancer compound, with its many biomolecular targets including the family of inflammatory cytokines, protein kinases, transcription factors, and anti-apoptotic proteins as well as individual targets such as COX-2, TNF and VEGF [18]. In this study, we report the efficacy of low-dose curcumin in inducing apoptosis in human melanoma cells (A375 and relatively resistant G361), and most surprisingly, its enhanced effect when combined at a low-dose, with the non-toxic drug, tamoxifen. More importantly, we show that this non-toxic combination prevents revival of chemo-resistant human melanoma cells, after drug removal. Interestingly enough, cell death upon combination may be a result of autophagy, and not primarily apoptosis. At advanced stages there is little to which melanoma responds as it is self-sufficient enough to maneuver past the cytotoxic insults of chemo-, radio- and immunotherapy. Here, we show that curcumin induces apoptosis in human melanoma cells in combination with the breast cancer drug tamoxifen and the effect is synergistic, with a possible occurrence of death by autophagy.

Previous studies have reported that curcumin has been completely ineffective in inducing apoptosis in human melanoma cells lines at doses of 50 μ M after 24 hours treatment [11]. Conversely in the same year, Bush *et al.* determined that curcumin could, in fact, induce apoptosis in melanoma cells at a 100 μ M dose,

independent of p53 status [12]. Furthermore, it was found that curcumin promoted both anti-proliferative and pro-apoptotic effects in melanoma cells at a lower dose of 10 μ M over a period of 96-120 hours treatment [13]. Siwak *et al*, also observed that curcumin-induced apoptosis in human melanoma was independent of the B-Raf/MEK/ERK signaling pathways [13].

Our studies show that cell viability of A375 human melanoma, does reduce upon curcumin treatment at a low dose of 10 μ M when cell viability is measured as a function of mitochondrial dehydrogenases using the WST-1 assay [Figure 1A]. Given that metabolic function of these dehydrogenases is disrupted, this suggests that mitochondria are targets of curcumin-induced apoptosis. Many studies indicate curcumin's ability to up-regulate pro-apoptotic proteins of the Bcl-2 family, down-regulate the anti-apoptotic members, dissipate the potential across the mitochondrial membrane leading to downstream caspase activation [26]. In this study, 10 μ M curcumin caused mitochondrial membrane permeabilization and dissipation of the potential across the mitochondrial membrane of A375 cells, and this occurred faster with co-treatment of curcumin and tamoxifen [Figure 6a]. Our finding is supported by a previous study, in which 10 μ M curcumin promoted rapid dissipation of the mitochondrial membrane potential in HeLa cells [27].

Upon further investigation, it was concluded that curcumin alone increased ROS generation from isolated A375 mitochondria when treated with curcumin [Figure 3]. This finding highlights curcumin's ability to not only act as an anti-oxidant but a pro oxidant. Earlier studies have proven curcumin's dichotomy as a

pro oxidant and an anti-oxidant. In a study by Kelly and co-workers on Jurkat T-lymphocytes, curcumin's pro oxidant activity was evident once it failed to not only prevent DNA damage (breaks in the DNA), but in fact was the cause of it [28]. In agreement with our findings, it has been speculated that below 50 μ M, curcumin acts as a pro-oxidant *in vitro* in transformed cells, resulting in increased ROS production, mitochondrial swelling, dissipation of the mitochondrial membrane potential, disruption of redox homeostasis and release of cytochrome *c* [9, 26]. We have also found that at concentrations below 50 μ M curcumin was able to destabilize A375 mitochondria, and activate caspase-9 (downstream of the mitochondria) (data not shown). As mentioned before, tamoxifen has been shown to not only act upon the estrogen receptor, but directly upon the mitochondria. Upon combinatorial treatment with tamoxifen on isolated mitochondria 5 μ M curcumin results in generation of excessive levels of ROS compared to either treatment alone [Figure 6B]. Upon addition of 10 μ M curcumin to tamoxifen this signal becomes saturated. Tamoxifen alone destabilizes the mitochondria to generate ROS, which agrees with previous studies that state that the drug can directly target the mitochondria [14, 15]. In a previous study the mitochondria of melanoma A375 cells has been shown to be vulnerable to tamoxifen treatment, thereby sensitizing these cells to co-treatment [16].

In this study, Normal Human Fibroblasts (NHFs) have been used as the normal, non-cancerous counterpart to human melanoma cells, and subjected to curcumin and/or tamoxifen treatment for 96 hours. Interestingly, Hoechst staining reveals that after this long-term treatment, NHFs remained unaffected by the either

singular treatments or even the co-treatment [Figure 9A]. Humans can tolerate a maximum of 12g of curcumin/day, proving that daily dietary quantities are non-toxic [29]. Tamoxifen has proven non-toxic to normal, non-cancerous cells in previous studies [15, 16]. The non-apoptotic A375 cells remaining after 96 hours of curcumin and tamoxifen co-treatment, retained the signals to commit suicide even after the drug was removed from fresh media, in which the cells were re-plated [Figure 10A and B]. Therefore, the ability of the co-treatment to prevent recovery i.e. permanently induce signals for cell death, makes this combination a potential non-toxic, effective therapeutic candidate.

Curcumin has also been shown to induce autophagy in cancer cells. For example, Human malignant glioma cells (U-373-MG and U87-MG) treated with curcumin at a concentration of 40 μ M initiated formation of autophagic vacuoles and this response was determined to be pro-death rather than pro-survival [24]. Autophagy is classified as programmed cell death II (PCD II) and is paradoxically linked to cancer, promoting both death and survival of the tumour cells. To limited application of cellular stress, cells (normal and cancerous) will use autophagy to adapt to the microenvironment. There are a number of anti-cancer therapies (i.e. doxorubicin, IFN γ , histone deacetylase inhibitors, rapamycin, and etoposide) that unintentionally induce pro-survival autophagy, thought to be responsible for chemo-resistance of tumor cells [30]. When too much stress is applied there is a massive autophagic response leading to autophagic cell death [17, 30]. Tamoxifen is a known inducer of autophagy. We show that tamoxifen treatment alone at 10 μ M induces an autophagic response in A375 melanoma cells [Figure 11A and B].

However, tamoxifen treatment alone is not enough to induce apoptosis in these cells [Figure 5a and b]. It could be inferred that upon combination with 10 μ M curcumin there may indeed be a massive autophagic response generated that could result in autophagic cell death. Qadir *et al.* have previously shown that 5 μ M tamoxifen is enough to incite pro-survival autophagy in MCF-7 breast cancer cells [31]. Addition of curcumin to tamoxifen most likely tips the balance from pro-survival to pro-death, and it is possible that autophagic death maybe the cause of such a high percentage of cell death in A375 cells [Figure 5A and B].

In conclusion, curcumin alone can kill chemo-resistant human melanoma cells through apoptosis; however, its effect is much greater at a lower dose through the combinatorial treatment with the breast cancer drug, tamoxifen, and this effect is non-toxic to normal, non-cancerous cells. Furthermore, the percentage of dead cells is not only attributed to apoptosis induction but possibly autophagy. How this form of programmed cell death is involved in this process is unclear although both curcumin and tamoxifen are known to induce autophagy. Given this evidence, non-toxic curcumin and tamoxifen are worthy of future clinical trials against chemo-resistant human malignant melanoma.

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Chapter 5

Conclusions & Future Prospectives

Conclusion

In conclusion, this thesis presents results that indicate potential of new natural chemotherapies for chemo-resistant melanoma. Pancratistatin (PST- from *Hymenocallis littorale*), curcumin (from turmeric- *Curcuma longa*) and dandelion root extract (DRE), were all effective in inducing apoptosis in melanoma cells. Interestingly, combining tamoxifen (TAM-estrogen receptor antagonist) to PST and curcumin elicited a synergistic cell death response in these cells. Initial work on the mechanism of cell death indicated that these compounds might be working through mitochondrial destabilization in cancer cells.

PST has been shown to induce apoptosis in a variety of cancers including neuroblastoma, breast carcinoma, leukemia and colon cancer [1]. The effect of PST on human melanoma has been studied here for the first time. PST was able to induce apoptosis in melanoma cells while remaining non-toxic to non-cancerous cells. Similar to a previously reported hypothesis, PST targets the mitochondria of melanoma cells. When PST is combined with tamoxifen there is a synergistic increase in apoptosis induction confirming the mitochondrial targeting of both drugs. We must note that TAM does not solely induce apoptosis in combinatorial treatments but also autophagy (see Chapter 4). A previous study on combinatorial treatment of PST and tamoxifen on breast cancer (MCF-7) cells [2] was the basis for a subsequent study involving the combination of narciclasine, a structural analog of PST, and TAM on MCF-7 cells. In this study, narciclasine proved to be less effective than PST when combined with TAM. Though a seemingly negative result, it may be

useful in determining which groups in the pharmacophore region of PST are crucial for effective induction of cell death in cancer cells. The mechanism by which PST specifically induces apoptosis in melanoma and other cancer cells is yet to be elucidated. In conclusion, human melanoma cell lines are susceptible to PST treatment; PST does target the mitochondria of melanoma cells.

Studies correlating diet, disease and populations are opening doors for the discovery of different herbs, spices and other natural sources and products used in ethnic and traditional medicines. Compounds like curcumin, which reportedly aims at a barrage of targets, may tackle cancer from more than one side. Targets include Bcl-2, Bid, IAPs, p53 proteins and pathways involved in inflammation, apoptosis, proliferation, cell survival as well as oxidative stress [3]. Unfortunately, the exact mechanism of action of curcumin in cancer cells is not clearly understood. In this study, curcumin was shown to be effective at significantly lower doses compared to other reports that have been performed with curcumin and cancer cells, and also in melanoma [4-8]. Furthermore, a novel combination of two non-toxic compounds - curcumin and tamoxifen- has been clearly shown to induce synergistic levels of apoptosis in chemo-resistant human melanoma cells. Dandelion Root Extract (DRE) has shown much promise in terms of its potential for development from bench-top to bedside. We are intrigued by the rapid and effective activity of DRE against chemo-resistant melanoma cells, especially considering it was found to be non-toxic to normal cells. It may be possible that the anti-cancer activity of DRE relies on the multi-compound nature of the extract; the various components of DRE might work together to perform as a whole to induce apoptosis in melanoma cells. An important

observation of this work was the effect of metformin (anti-type II diabetes drug) on the relatively resistant G361 melanoma cell line when used in combination with DRE. This metabolism-interfering compound successfully sensitized G361 cells to the effects of DRE; however, it is poorly understood how metformin acts on cancer cells.

Future Prospectives

Pancratistatin and tamoxifen

The primary obstacle regarding this potential therapy is that PST is difficult to produce in bulk quantities. An *in vivo* model using B16 murine melanoma should be analyzed to assess the efficacy of co-treatment with PST and tamoxifen on human melanoma cells in a living system. The target of PST must be determined and the mechanism by which it induces apoptosis must also be elucidated. One way of doing this would be to radio-label PST and analyze its uptake and its interactions with its cellular target in mitochondria. Synthetic derivatives of PST should be analyzed for activity and compared to PST, and also their potential for combinatorial treatment with tamoxifen. Lastly, a new technology, Phenotype MicroArrays™ (a metabolic array by Biolog Inc), can be used in futures studies to simultaneously compare thousands of co-regulated genes or proteins that differ in say human melanoma cells vs. non-cancerous cells. It will also be able to show the effect of drugs on genes/proteins on the cells.

Dandelion Root Extract

Further work with DRE involves the isolation of its different components, and testing each separately on human melanoma cells to observe their efficacy *in vitro*. It is plausible that one or more components of DRE might be working synergistically with one another to produce the observed effect of programmed cell death in human melanoma cells. Components of DRE include sesquiterpenes (derivatives of hermacranolide, eudesmanolide and guaianolide); different triterpenes like taraxasterol- their hydroxy derivatives and their acetates; phenolics compounds (such as chicoric acid, vanillic acid, p- hydroxyphenylacetic acid, p-hydroxybenzoic acid, syringic acid, caffeic acid, chlorogenic acid and ferulic acid); and coumarins (scopolectin, esculectin and umbelliferone) [9]. Knowledge of these components, and the fact that our extract is water-based, can help us determine which are the active anti-cancer components in DRE. Therefore, individual and different sets of compounds can be used to determine which group of components provides greatest anti-cancer potential against human melanoma and other cancers. We also need to determine at which subcellular level these components are acting. Therefore treated melanoma cells should be separated into different subcellular fractions after which the absorbance of each component in that fraction can be measured, using spectrophotometry or even high-performance liquid chromatography (HPLC). This is based on the fact that each compound in DRE should absorb UV light at a specific

wavelength and therefore can be detected if a certain subcellular fraction absorbs at that wavelength. Phenotype MicroArrays™ can be used to analyze the effect of DRE-treated melanoma cells vs. non-cancerous cells. Needless to say, *in vivo* work should be carried out using animal models to gauge the clinical potential of this herbal extract.

Curcumin and tamoxifen

In the same vein as PST and tamoxifen, future prospects with curcumin also involve the examination of its targets in human melanoma cells. This could be performed by radio-labeling curcumin (using either C¹³ or tritium labeled hydrogen of hydroxyl groups – responsible for conferring antioxidant activity of curcumin [10]) and analyzing its uptake and location of its cellular interactions. *In vivo* studies are also required, not only to determine the effect of curcumin and tamoxifen, but also to study the bioavailability of curcumin, since curcumin is known to have poor bioavailability. Furthermore, it would be interesting to observe the effect of curcumin and tamoxifen on other skin cells (*in vivo*) like keratinocytes (even in the presence of melanoma- a co culture), this could be done using the WST-1 assay, Hoechst staining or even Flow cytometry. Keratinocytes are known to support melanoma cells by providing growth factors to sustain them [11], therefore their effect on these cells in co-culture would provide better understanding of how effective the curcumin and tamoxifen co-treatment is on the epidermal system as a whole. This study could further be extended using Phenotype MicroArrays™ to compare curcumin and/or tamoxifen treated melanoma cells to non-cancerous

counterparts, and other skin cells that support melanocytic growth. Development of topical applications of curcumin and tamoxifen as oil-based creams should also be a future prospect.

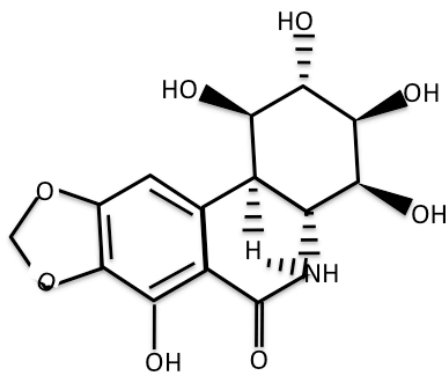
These results represent a novel window of opportunity for the development of natural products for chemo-resistant melanoma.

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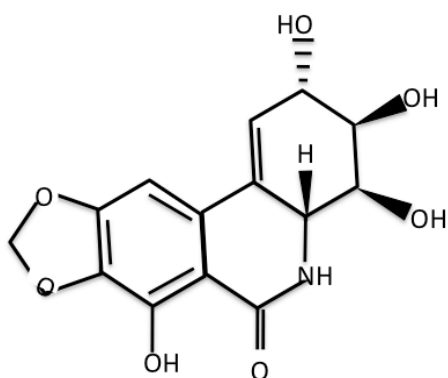
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Appendix

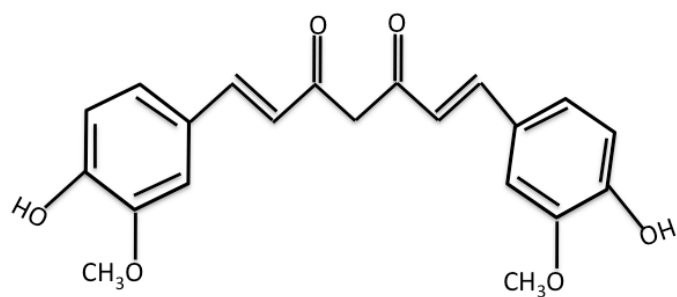
Pancratstatin



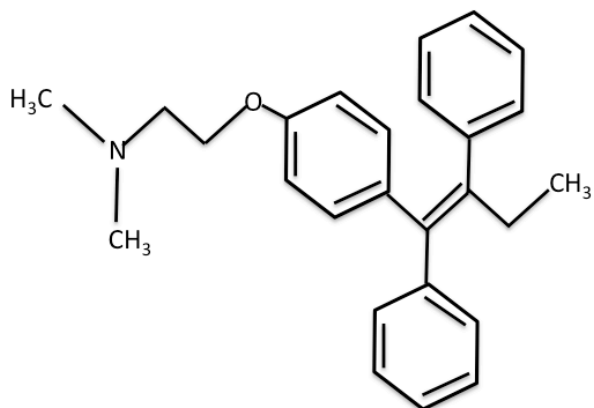
Narciclasine



Curcumin

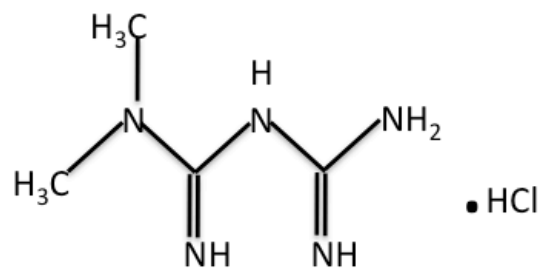


Tamoxifen



Metformin

Metformin is expressed in its hydrochloride form (Metformin hydrochloride).



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